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A Review: On the Mechanism of Action of Certain Temporary Anticancer Agents

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It has been said that "the bibliographer in cancer research is confronted with a monstrous and amorphous literature, controversial, often hastily ambitious, and frequently haunted by the ghosts of countless once-hopeful and dazzling hypotheses" (20).

It is not the purpose of this short review to provide another ghost, but rather to point to certain experimental results which suggest that there may be a degree of similarity in the actions of certain of the heterogeneous group of agents which possess unequivocal temporary anticancer activity in experimental animals.

There is as yet little convincing evidence regarding the mechanisms by which certain substances provide slight cytotoxic specificity for certain neoplastic tissue. It is sometimes assumed that the known cytotoxic anticancer agents preferentially damage rapidly metabolizing and dividing cells on a "quantitative differential" basis. Such an explanation fails to explain the lack of universality of certain tumor-inhibiting compounds. Also, the development of drug-resistant and drug-dependent strains of mouse leukemia (11, 30) suggests that "qualitative" differences may exist within a given population of neoplastic cells (28). "Qualitative" differences in the catch-all sense intended refer to obscure differences somewhere in the conglomeration of simultaneous chemical events leading to growth in the normal and neoplastic cell. It would, in fact, be difficult, without reverting to some refuge haunted by vital forces, to visualize the causes of the quantitative differentials (rate differences) between normal and neoplastic cells without assuming some basic qualitative differences, not necessarily in the better known metabolic pathways but perhaps within catalytic or rate-determining systems or templates thereof.

Rather extensive studies have now been carried out with regard to the gross anatomical distribution of labeled chemotherapeutic agents in mice with various neoplasms. No evidence has been obtained which suggests that radioactive atoms from the following compounds are preferentially localized in the cancer cells which they inhibit: carbonyl-labeled urethan (38), methylene-labeled urethan (38), methyl-labeled nitrogen mustard (HN2) (39), 8-azaguanine-2-C^{14} (4), 2,6-diaminopurine-2-C^{14},[1] ring-labeled triethylene melamine,[2] and I^{131}-labeled iodo-A-methopterin.[3] Nor have data yet been obtained which suggest that these agents localize (anatomically) in some particular organ or tissue and influence neoplastic growth by secondary effects.

Since the observed alterations in the metabolism of neoplastic cells are quantitative, it becomes important to consider the systems which "direct" the synthesis of cellular catalysts (enzymes). Control of the specificity of enzymes is now generally attributed to genes. It is possible that each gene imparts to the protein portion of an enzyme a composition and configuration essential to the action of that enzyme. Genes are now often considered as nuclear or cytoplasmic nucleic acid

[1] H. E. Skipper and C. C. Stock, unpublished data.

[2] H. E. Skipper, L. L. Bennett, Jr., M. Bell, and L. Simpson, unpublished data.

[3] J. A. Johnson, Jr., and H. E. Skipper, unpublished data.

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containing substances concerned with hereditary transmission of biochemical characteristics.

Such nonspecific reasoning suggests that it is perhaps not too illogical to consider the metabolism of a possible gene moiety, nucleic acids, and chemically related biologic substances (nucleotides) as sites of primary action of certain temporary anticancer agents.

However, in order to maintain a degree of objectivity, it should be pointed out that to date there is no direct and unequivocal evidence as to the role of nucleoprotein macromolecules in protein or enzyme synthesis. The importance ascribed to nucleic acids in self-duplication processes appears to be the result of *a priori* reasoning and association.

In the discussion that follows, an attempt has been made to review briefly certain experimental results relating the biochemical effects of a number of temporary anticancer agents to nucleotide metabolism. Space does not permit even a brief review of the rapidly expanding current literature on nucleotide metabolism which is relevant to this discussion; rather, an attempt has been made to include only references having to do with the effects of certain anticancer agents on these events.

Microbial Studies

One of the most useful procedures for elucidating the site of the action of a growth inhibitor is inhibition analysis employing a suitable biological system. If it can be demonstrated that a metabolite prevents the growth inhibitory action of an agent, it is generally assumed that the inhibitor is affecting a series of chemical events in which the metabolite is involved.

A considerable fund of knowledge has been accumulated on the prevention of the growth inhibitory effects of temporary anticancer agents in bacteria. Certain significant results on this subject are summarized in Table 1.

Reversal of the Tumor Inhibiting Action of Certain Anticancer Agents

Perhaps the most convincing manner of demonstrating the site of action of a compound which inhibits the growth of an experimental tumor is the simultaneous administration of a metabolite which will prevent or reverse the tumor inhibition.

With perhaps one exception (A-methopterin), it has been impossible to achieve complete prevention of the anticancer activity of the well known tumor-inhibiting agents by administration of candidate reversal agents. This difficulty is understandable when one considers the heterogeneous milieu which both antagonist and metabolite pass through en route to neoplastic cells in the intact animal. However, the data obtained to date, although not so extensive as those obtained in micro-organisms, are of considerable interest (Table 2).

TABLE 1

REVERSAL OF THE GROWTH-INHIBITORY EFFECTS OF CERTAIN ANTICANCER AGENTS IN MICROBIAL SYSTEMS

Anticancer agent	Reversal agent	Microbial system	Ref.
4-Aminopteroylglutamic acids	Folic acid	*S. faecalis* R	36
	Citrovorum factor	*S. faecalis* R	6
	Thymidine	*L. citrovorum*	35
	Thymine+purines	*S. faecalis* R	*
	Cortisone	*L. citrovorum*	16
Urethan	2,6-Diaminopurine	*E. coli*	48
	2,6-Diaminopurine riboside	*E. coli*	48
Formamide†	2,6-Diaminopurine	*E. coli*	‡
	2,6-Diaminopurine riboside	*E. coli*	‡
2,6-Diaminopurine	Adenine, guanine, hypoxanthine Purine ribosides, and ribonucleotides	*L. casei*	12
	Folic acid	*L. casei*	25
8-Azaguanine	Guanine	*Tetrahymena geleii*	26
2,4-Diaminopyrimidines	Folic acid Purines	*L. casei*	13

* G. P. Wheeler, unpublished data.

† This compound has been shown to inhibit Sarcoma 180 (C. C. Stock, personal communication).

‡ H. E. Skipper, F. M. Schabel, Jr., G. P. Wheeler, J. C. Garlington, and J. R. Thomson, unpublished data.

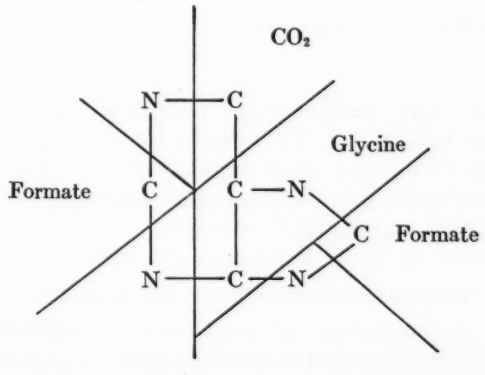
The Effects of Anticancer Agents on Viruses

This is not a proper place to invoke the argument as to the possible virus nature of neoplasia. However, it is well known that virus particles contain varying amounts of nucleoprotein, as do chromosomes and probably genes; therefore, it is of possible interest to summarize certain data on the antiviral activity of several anticancer agents (Table 3).

EFFECTS OF CERTAIN ANTICANCER AGENTS ON *de novo* NUCLEIC ACID SYNTHESIS

Tracer technics have made possible studies of the dynamics of anabolic and catabolic processes in the intact animal and the effects of therapy on such processes.

Using C^{13}, Buchanan and his associates (8, 49) demonstrated that formate, glycine, and CO_2 are precursors of the carbon atoms of the purine skeleton of uric acid:



The same compounds apparently serve as precursors for the nucleic acid purines (50). With this knowledge it has been possible to study the effects of anticancer agents on *de novo* nucleic acid purine synthesis by the direct procedure of measuring incorporation of the labeled precursors in control and treated animals. The specificity of inhibitions observed can be roughly ascertained by simultaneously measuring incorporations of labeled formate or carbon dioxide into protein or whole tissue.

A considerable amount of work of this nature has now been reported. Table 4 summarizes certain of these results. It should be clearly understood that the specific chemical events affected in these inhibitions are not demonstrated by such experiments. It cannot be assumed, for instance, because x-radiation inhibits formate incorporation into nucleic acid purines and not protein, that the final step in closing the purine ring (formylation) is the site of action of ionizing radiation. It is obvious that any of a number of blocks in the energy-coupling processes, precursor-producing reactions, or catalytic events could provide for such slowing down of *de novo* nucleic acid synthesis.

TABLE 2

PREVENTION OF THE ANTICANCER ACTIVITY OF CERTAIN AGENTS

Anticancer agent	Reversal agent	Type of neoplasia	Ref.
A-Methopterin	Folic acid	Ak4 leukemia	10, 42
	Citrovorum factor	Ak4 leukemia	9
	DNA (partial)	Ak4 leukemia	37
	B_{12} (partial)	Ak4 leukemia	43
8-Azaguanine	Adenine, purine, neucleosides and purine nucleotides	Brown-Pearce carcinoma	*
	Guanylic acid (partial)	Leukemia 1210	17

* A. Gellhorn, personal communication.

TABLE 3

THE EFFECTS OF CERTAIN TEMPORARY ANTICANCER AGENTS ON VIRUSES

Anticancer agent	Virus inhibited	Remarks	Ref.
A-Methopterin	Rous sarcoma	Feline pneumonitis, polio, influenza, vaccinia, and Western equine encephalomyelitis unaffected *in vivo*	*
2,6-Diaminopurine	Russian spring and summer encephalitis	Feline pneumonitis, polio, influenza, vaccinia, and Western equine encephalomyelitis unaffected *in vivo*	15, *
8-Azaguanine	Tobacco mosaic	Feline pneumonitis, polio, influenza, vaccinia, and Western equine encephalomyelitis unaffected *in vivo*	32, *
Netropsin†	Vaccinia	Feline pneumonitis, polio, influenza, and Western equine encephalomyelitis unaffected *in vivo*	*
X-radiation	Many affected *in vitro*		
Nitrogen mustard	Many affected *in vitro*	Feline pneumonitis, polio, influenza, vaccinia, and Western equine encephalomyelitis unaffected *in vivo*	22, *

* F. M. Schabel, Jr., unpublished data.

† Netropsin, an antibiotic from *Streptomyces netropsis* (14), was first observed to inhibit intracerebrally inoculated vaccinia in mice,* and was then found to have slight to moderate inhibitory effects in several experimental animal tumors and leukemias (H. E. Skipper, M. Bell, J. R. Thomson, and F. M. Schabel, Jr., unpublished data).

FIXATION OF CERTAIN ANTICANCER AGENTS TO NUCLEIC ACID MOIETIES

Several years ago it was reported that 8-azaguanine-2-C^{14} is fixed in small amounts, as such, in the nucleic acids of mouse viscera (33). This observation has been confirmed in a second series of experiments,[4] by similar technics by Mandel (31) and without the aid of tracer technics in *Tetrahymena geleii* by Heinrich (21).

Similar results have been obtained showing the fixation of 2,6-diaminopurine-2-C^{14} as such, in mouse nucleic acids.[4] In addition, it has been demonstrated that 2,6-diaminopurine-2-C^{14} is converted *in vivo* to 2,6-diaminopurine riboside in considerable quantity. This diaminopurine ribo-

[4] L. L. Bennett, Jr., H. E. Skipper, J. Meade, J. E. Hill, and L. Simpson, unpublished data.

side was first isolated in the ATP-fraction and is believed to be a phosphorylated nucleoside, since on pyridine hydrolysis, paper chromatography, and autoradiography a 2,6-diaminopurine riboside autoradiogram was obtained which was confirmed by co-chromatography with known compounds (52).

Results of a similar nature have also been obtained by Weygand *et al.*, showing fixation of evidence that incorporation of "unnatural" bases into nucleotides or reaction of nitrogen mustard with nucleotide bases are not impossible means for explaining the biologic actions of these agents.

More pertinent data have recently been obtained which show that 8-azaguanine-2-C^{14} is incorporated into the RNA of an 8-azaguanine-susceptible strain of leukemia (L1210) at levels of 100 times the incorporation of this purine antagonist into 8-azaguanine-dependent leukemia (L1210-8AG-D) (3). This could be considered as suggestive evidence that fixation of 8-azaguanine in nucleic acids may be related to the cytotoxic action of this compound.

TABLE 4

EFFECTS OF CERTAIN ANTICANCER AGENTS ON INCORPORATION OF PRECURSOR MOLECULES INTO WHOLE TISSUES, PROTEIN AND NUCLEIC ACID MOIETIES

Labeled precursor employed	Anticancer agent	Period of exper. (hrs.)	Tissue	Protein or whole tissue	Combined nucleic acids	DNA	RNA	Ref.
Formate-C^{14}	Aminopterin	6	Viscera	0	+++			45
Formate-C^{14}	A-methopterin	6	Viscera	0	+++			45
Formate-C^{14}	A-methopterin	6	Viscera			+++	+++	47
Formate-C^{14}	Aminopterin	24	Intestine			+++	++	19
Formate-C^{14}	Aminopterin	24	Liver			++	+	19
Adenine-C^{14}	Aminopterin	24	Intestine			+	0	19
Formate-C^{14}	A-methopterin	6	Leukemic livers and spleens (Ak4 leukemia)	0	+++			41
Formate-C^{14}	A-methopterin	6	Leukemia L1210 (A-methopterin-susceptible)	++		+++	+++	40
Formate-C^{14}	A-methopterin	6	Leukemia L1210 (A-methopterin-dependent)	S		S	S	40
Acetate-C^{14}*	X-radiation	6	Intestinal mucosa	0		++		23
Acetate-C^{14}*	X-radiation	6	Liver	S		+		23
Acetate-C^{14}*	X-radiation	6	Kidney	0		++		23
C^{14}-O_2	X-radiation	6	Viscera	S		+		44
Formate-C^{14}	X-radiation	6	Viscera	S	++			44
Glycine-1-C^{14}	X-radiation	5	Bone marrow	0		+++	+++	1
Glycine-1-C^{14}	X-radiation	5	Intestine	0		+++	+++	1
Formate-C^{14}	Nitrogen mustard	24	Intestine			++	0	18
Formate-C^{14}	Nitrogen mustard	6	Viscera	S	+			46
Formate-C^{14}	Nitrogen mustard	6	Viscera	S		++	?	†
Formate-C^{14}	Urethan	6	Viscera	S	+			46
Formate-C^{14}	8-Azaguanine	6	Viscera	0		+	+	†
Formate-C^{14}	8-Azaguanine	6	Viscera	0	++			46
Formate-C^{14}	2,6-Diaminopurine	6	Viscera	S	++			46
Formate-C^{14}	Cortisone	6	Viscera	0	+			46

NOTE: S = stimulation
0 = no significant effects (± 25 per cent of control)
+ = inhibition of 25–50 per cent
++ = inhibition of 50–75 per cent
+++ = inhibition of 75 per cent

* This study was probably a measure of incorporation of $C^{14}O_2$ resulting from oxidation of acetate-C^{14}, since acetate is not a precursor of purines.
† L. L. Bennett, Jr., and H. E. Skipper, unpublished data.

Br- labeled bromouracil, a thymine antagonist, in bacterial nucleic acids (51). Bromouracil is not known to inhibit tumors.

Wheeler[5] has carried out *in vitro* and *in vivo* experiments which demonstrate that methyl-labeled nitrogen mustard is fixed to nucleic acid purines and pyrimidines, and that purine and pyrimidine-nitrogen mustard reaction products can be isolated by paper or ion-exchange chromatography.

Such studies as are mentioned above are not conclusive and should only be considered as

[5] G. P. Wheeler, unpublished data.

DRUG RESISTANCE IN MOUSE LEUKEMIAS

The development of resistance to inhibiting agents by neoplastic tissue seems to be a universal occurrence. Drug resistance in micro-organisms has been known for many years and has in certain instances been rather conclusively ascribed to chemical selection of existing drug-resistant mu-

tants in a bacterial population. Evidence has been recently obtained which strongly suggests that the development of resistance in mouse leukemia is the result of a similar series of events (28).

Burchenal and Law have developed, by treated transplantation, strains of mouse leukemia which are resistant to A-methopterin (9, 30). Law has succeeded in developing strains of L1210 leukemia which are dependent on A-methopterin (30) and 8-azaguanine,[6] respectively, for optimal growth.

A-methopterin-dependent L1210 leukemia is not stimulated by folic acid or citrovorum factor. In fact, citrovorum factor partially prevents the growth-stimulating action of A-methopterin in the dependent leukemia (29). These data suggest that this leukemia does not convert A-methopterin into folic acid or citrovorum factor.

It has been found that A-methopterin profoundly inhibits *de novo* synthesis of DNA and RNA purines of A-methopterin-susceptible leukemic cells and viscera of mice bearing this leukemia. However, administration of A-methopterin more than doubles the rate of *de novo* DNA and RNA synthesis in the A-methopterin-dependent leukemic cells while profoundly inhibiting nucleic acid synthesis in the viscera of mice bearing this tumor (40).

It has already been mentioned that 8-azaguanine is fixed in the RNA of 8-azaguanine-susceptible leukemic cells at levels 100 times that found in the RNA of dependent leukemic cells.

Such observations as have been made to date on the metabolism of drug-susceptible and resistant or dependent neoplastic tissue suggest that differences in nucleic acid metabolism may exist. In the case of the drug-dependent leukemias it seems possible that "normal" metabolites may be acting as antagonists and that the anticancer agents may antagonize these "normal" antagonists to the benefit of the dependent neoplastic cells.

Hirschberg *et al.* have made the extremely interesting observation that experimental tumors which are most susceptible to 8-azaguanine have the lowest concentration of a deaminase which converts 8-azaguanine to 8-azaxanthine (24), suggesting that the variation in ability of neoplastic cells to detoxify an antagonist may in some instances account for variations in response to anticancer agents.

The field of drug-resistance (natural or induced) in cancer would appear to be one of greatest theoretical and practical importance.

[6] L. W. Law, personal communication.

CURRENT HYPOTHESES

A review on the present subject is undoubtedly premature, since detailed information on the mode of action of the temporary anticancer agents is not yet at hand. The data summarized in the tables presented above suggest that a pattern of action on one complicated biochemical system may exist among the heterogeneous agents which inhibit tumor growth.

In conclusion, it may be worth while to mention briefly certain current views on the possible biochemical actions of several classes of anticancer agents. These hypotheses must be considered for what they are, logical guesses based largely on information gained from studies on normal metabolism. Such hypotheses must withstand the test of time and must eventually be expanded to explain the reasons for the slight temporary cytotoxic specificity exhibited by anticancer agents for certain neoplastic cells.

The *4-aminopteroylglutamic acids* almost surely inhibit leukemia by antagonism of folic acid or citrovorum factor, since either of these compounds will prevent the antileukemic action of this class of agent. It appears that folic acid is converted to citrovorum factor which is associated with enzymes having to do with transfer of single carbon units. Aminopterin (4-aminopteroylglutamic acid) prevents conversion of folic acid to citrovorum factor (34), and compounds of this class compete with formed citrovorum factor. The end result of this antagonism is an inhibition of nucleic acid synthesis as well as other biochemical processes. It seems probable that the primary mechanism by which the 4-aminopteroylglutamic acids provide antileukemic activity is through inhibition of nucleotide synthesis.

The *purine antagonists*, 2,6-diaminopurine and 8-azaguanine, inhibit certain experimental tumors and leukemias *in vivo*. Both of these agents are fixed as such in mammalian nucleic acids. Diaminopurine acts as a precursor of nucleic acid guanine (2)[4] and is converted to diaminopurine ribosephosphate (52). It is possible that diaminopurine ribosephosphate is acting as an antagonist of adenine containing coenzymes (7). 8-Azaguanine may inhibit cell growth by formation of "fraudulent" nucleotides but proof of this hypothesis is still lacking.

There are data which suggest that alkylating agents of the *nitrogen mustard* class react irreversibly with nucleotide bases. Such chemical reactions could account for chromosome malfunctions so dramatically demonstrated by the cytologist and the geneticist.

The effective reversal of *urethan* toxicity to *E.*

coli by 2,6-diaminopurine or 2,6-diaminopurine riboside would appear to relate the action of this anticancer agent to nucleotide metabolism. Also, it has been reported that thymine partially neutralizes the chromosome-damaging action of urethan (5), suggesting that this agent may inhibit transmethylations involved in desoxyribonucleic acid thymine synthesis.

The mechanism by which *ionizing radiation* produces biologic effects, including tumor inhibition, is becoming a specialized field of study in itself. In keeping with the present discussion, it is of interest to note that it has now been reported from three different laboratories (1, 23, 44) that nucleic acid purine synthesis in mammals is profoundly inhibited at 6 hours after treatment with x-radiation and at this time no inhibition of acetate, glycine or formate carbon incorporation into protein is observed.

CONCLUSIONS

This review on the possible sites of biochemical action of certain temporary anticancer agents is necessarily superficial and incomplete and has been directed toward points which seem important to the reviewer. Many aspects of the subject as well as alternative hypotheses have been ignored.

It is not intended to suggest that the present fad of blaming the effects of carcinostatic, carcinogenic, and mutagenic agents on their seeming disruption of nucleotide metabolism may not eventually join the "ghosts of countless once-hopeful and dazzling hypotheses (20)."

However, the evidence that certain of the heterogeneous group of agents which temporarily affect neoplastic growth also affect chemical events involved in nucleotide metabolism would seem to emphasize the necessity of attempting to obtain more basic knowledge in this field of biochemistry, if for nothing more than to lay to rest the growing assumption that here lies the core of the cancer problem.

REFERENCES

1. Abrams, R. Effect of X-rays on Nucleic Acid and Protein Synthesis. Arch. Biochem. & Biophys., **30**:90–99, 1951.
2. Bendich, A., and Brown, G. B. 2,6-Diaminopurine, a Precursor of Nucleic Acid Guanine. J. Biol. Chem., **176**: 1471–72, 1948.
3. Bennett, L. L., Jr.; Skipper, H. E.; and Law, L. W. Incorporation of 8-Azaguanine-2-C^{14} into 8-Azaguanine-susceptible and 8-Azaguanine-dependent Leukemic Tumors. Fed. Proc., **12**:300–301, 1953.
4. Bennett, L. L., Jr.; Skipper, H. E.; Mitchell, J. H., Jr.; and Sugiura, K. Studies on the Distribution of Radioactive 8-Azaguanine (Guanazolo) in Mice with E 0771 Tumors. Cancer Research, **10**:644–46, 1950.
5. Boyland, E. Different Types of Carcinogens and Their Possible Modes of Action: *A Review*. Cancer Research, **12**:77–84, 1952.
6. Broquist, H. P.; Stokstad, E. L. R.; and Jukes, T. H. Comparative Biological Activity of Crystalline Citrovorum Factor and Pteroylglutamic Acid. Fed. Proc., **10**:167, 1951.
7. Brown, G. B. Symposium for Fundamental Cancer Research, Houston, Texas, 1952.
8. Buchanan, J. M.; Sonne, J. C.; and Delluva, A. M. Biological Precursors of Uric Acid. II. The Role of Lactate, Glycine, and Carbon Dioxide as Precursors of the Carbon Chain and Nitrogen Atom 7 of Uric Acid. J. Biol. Chem., **173**:81–98, 1948.
9. Burchenal, J. H.; Johnston, S. F.; Burchenal, J. R.; Kushida, M. N.; Robinson, E.; and Stock, C. C. Chemotherapy of Leukemia. IV. Effect of Folic Acid Derivatives on Transplanted Mouse Leukemia. Proc. Soc. Exper. Biol. & Med., **71**:381–87, 1949.
10. Burchenal, J. H.; Kushida, M. N.; Johnston, S. F.; and Cremer, M. A. Prevention of the Chemotherapeutic Effects of 4-Amino-N^{10}-Methyl-pteroylglutamic Acid on Mouse Leukemia by Pteroylglutamic Acid. Proc. Soc. Exper. Biol. & Med., **71**:559–62, 1949.
11. Burchenal, J. H.; Robinson, E.; Johnston, S. F.; and Kushida, M. N. The Induction of Resistance to 4-Amino-N^{10}-Methylpteroylglutamic Acid in a Strain of Transmitted Mouse Leukemia. Science, **111**:116–17, 1950.
12. Elion, G. B., and Hitchings, G. H. Antagonists of Nucleic Acid Derivatives. IV. Reversal Studies with 2-Aminopurine and 2,6-Diaminopurine. J. Biol. Chem., **187**:511–22, 1950.
13. Falco, E. A.; Hitchings, G. H.; Russell, P. B.; and VanderWerff, H. Antimalarials as Antagonists of Purines and Pteroylglutamic Acid. Nature, **164**:107–8, 1949.
14. Finlay, A. C.; Hochstein, F. A.; Sobin, B. A.; and Murphy, F. X. Netropsin: A New Antibiotic Produced by Streptomyces. J. Am. Chem. Soc., **73**:341–43, 1951.
15. Friend, C. Effect of 2,6-Diaminopurine on Virus of Russian Spring-Summer Encephalitis in Tissue Culture. Proc. Soc. Exper. Biol. & Med., **78**:150–53, 1951.
16. Gaines, D. S., and Broquist, H. P. Microbiological Activity of Cortisone Acetate. Fed. Proc., **10**:186, 1951.
17. Goldin, A.; Greenspan, E. M.; and Schoenbach, E. B. Studies on the Mechanism of Action of Chemotherapeutic Agents in Cancer. IV. Relationship of Guanine and Guanylic Acid to the Action of Guanazolo on Lymphoid Tumors in Mice and Rats. J. Nat. Cancer Inst., **11**:319–38, 1950.
18. Goldthwait, D. A. Effect of Nitrogen Mustard on Nucleic Acid Metabolism. Proc. Soc. Exper. Biol. & Med., **80**: 503–5, 1952.
19. Goldthwait, D. A., and Bendich, A. Effects of a Folic Acid Antagonist on Nucleic Acid Metabolism. J. Biol. Chem., **196**:841–51, 1952.
20. Greenstein, J. P. Biochemistry of Cancer. New York: Academic Press, Inc., 1947.
21. Heinrich, M. R.; Dewey, V. C.; Parks, R. E., Jr.; and Kidder, G. W. The Incorporation of 8-Azaguanine into the Nucleic Acid of *Tetrahymena geleii*. J. Biol. Chem., **197**: 199–204, 1952.
22. Herriott, R. M. Inactivation of Viruses and Cells by Mustard Gas. J. Gen. Physiol., **32**:221–39, 1948.
23. Hevesy, G. Effects of X-rays on the Incorporation of C^{14} into Desoxyribonucleic Acid. Nature, **163**:869–70, 1949.
24. Hirschberg, E.; Kream, J.; and Gellhorn, A. Enzymatic Deamination of 8-Azaguanine in Normal and Neoplastic Tissue. Cancer Research, **12**:524–28, 1952.
25. Hitchings, G. H.; Elion, G. B.; VanderWerff, H.;

and FALCO, E. A. Pyrimidine Derivatives as Antagonists of Pteroylglutamic Acid. J. Biol. Chem., **174**:765–66, 1948.

26. KIDDER, G. W.; DEWEY, V. C.; PARKS, R. E., JR.; and WOODSIDE, G. L. Purine Metabolism in *Tetrahymena* and Its Relation to Malignant Cells in Mice. Science, **109**:511–14, 1949.
27. KORNBERG, A., and PRICER, W. E., JR. Enzymatic Phosphorylation of Adenosine and 2,6-Diaminopurine Riboside. J. Biol. Chem., **193**:481–95, 1951.
28. LAW, L. W. Origin of the Resistance of Leukemic Cells to Folic Acid Antagonists. Nature, **169**:628–29, 1952.
29. ———. Response of a Resistant Variant of Leukemic Cells to an Antagonist of Pteroylglutamic Acid. Proc. Soc. Exper. Biol. & Med., **77**:340–44, 1951.
30. LAW, L. W., and BOYLE, P. J. Development of Resistance to Folic Acid Antagonists in a Transplantable Lymphoid Leukemia. Proc. Soc. Exper. Biol. & Med., **74**:599–602. 1950.
31. MANDEL, H. G., and CARLO, P. E. Tumor Purine Metabolism as Studied with Radioactive Guanine and Azaguanine. Résumés des Communications, p. 475. Second International Congress of Biochemistry, Paris, July 21, 1952.
32. MATTHEWS, R. E. F. Effect of Purines on the Multiplication of Plant Viruses. Nature, **169**:500–501, 1952.
33. MITCHELL, J. H., JR.; SKIPPER, H. E.; and BENNETT, L. L., JR. Investigation of the Nucleic Acids of Viscera and Tumor Tissue from Animals Injected with Radioactive 8-Azaguanine. Cancer Research, **10**:647–49, 1950.
34. NICHOL, C. A., and WELCH, A. D. On the Mechanism of Action of Aminopterin. Proc. Soc. Exper. Biol. & Med., **74**:403–11, 1950.
35. SAUBERLICH, H. E. The Relationship of Folic Acid, Vitamin B_{12}, and Thymidine in the Nutrition of *Leuconostoc citrovorum* 8081. Arch. Biochem. & Biophys., **24**:224–32, 1949.
36. SEEGER, D. R.; SMITH, J. M., JR.; and HULTQUIST, M. E. Antagonists for Pteroylglutamic Acid. J. Am. Chem. Soc., **69**:2567, 1947.
37. SKIPPER, H. E.; BELL, M.; and CHAPMAN, J. B. Partial Reversal of the Anti-leukemic Action of Folic Acid Antagonists by Nucleic Acids. Cancer, **4**:357–59. 1951.
38. SKIPPER, H. E.; BENNETT, L. L., JR.; BRYAN, C. E.; WHITE, L., JR.; NEWTON, M. A.; and SIMPSON, L. Carbamates in the Chemotherapy of Leukemia. VIII. Overall Tracer Studies on Carbonyl-labeled Urethan, Methylene-labeled Urethan, and Methylene-labeled Ethyl Alcohol. Cancer Research, **11**:46–51, 1951.
39. SKIPPER, H. E.; BENNETT, L. L., JR.; and LANGHAM, W. H. Overall Tracer Studies with C^{14}-labeled Nitrogen Mustard in Normal and Leukemic Mice. Cancer, **4**:1025–27, 1951.
40. SKIPPER, H. E.; BENNETT, L. L., JR.; and LAW, L. W. Effects of A-Methopterin on Formate Incorporation into the Nucleic Acids of Susceptible and Resistant Leukemic Cells. Cancer Research, **12**:677–79, 1952.
41. SKIPPER, H. E., and BURCHENAL, J. H. The Nucleic Acid-inhibiting Action of 4-Amino-N^{10}-Methylpteroylglutamic Acid in Mice with a Sensitive and Resistant Strain of Leukemia. Cancer Research, **11**:229–31, 1951.
42. SKIPPER, H. E.; CHAPMAN, J. B.; and BELL, M. Studies on the Role of Folic Acid in the Leukemic Process. Cancer, **3**:871–73, 1950.
43. ———. Partial Reversal of the Antileukemic Action of Folic Acid Antagonists by Vitamin B_{12}. Cancer Research, **11**:161–63, 1951.
44. SKIPPER, H. E., and MITCHELL, J. H., JR. Effect of Roentgen-Ray Radiation on the Biosynthesis of Nucleic Acids and Nucleic Acid Purines. Cancer, **4**:363–66, 1951.
45. SKIPPER, H. E.; MITCHELL, J. H., JR.; and BENNETT, L. L., JR. Inhibition of Nucleic Acid Synthesis by Folic Acid Antagonists. Cancer Research, **10**:510–12, 1950.
46. SKIPPER, H. E.; MITCHELL, J. H., JR.; BENNETT, L. L., JR.; NEWTON, M. A.; SIMPSON, L.; and EIDSON, M. Observations on Inhibition of Nucleic Acid Synthesis Resulting from Administration of Nitrogen Mustard, Urethan, Colchicine, 2,6-Diaminopurine, 8-Azaguanine, Potassium Arsenite, and Cortisone. Cancer Research, **11**:145–49, 1951.
47. SKIPPER, H. E.; MORGAN, C.; and BENNETT, L. L., Jr. Effects of A-Methopterin (4-Aminopteroylglutamic Acid) on the Synthesis of the Adenine Moiety of Adenosine Triphosphate. Cancer Research, **12**:413–14, 1952.
48. SKIPPER, H. E., and SCHABEL, F. M., Jr. Reversal of the Growth Inhibitory Action of Urethan by 2, 6-Diaminopurine. Arch. Biochem. Biophys., **40**:476–78, 1952.
49. SONNE, J. C.; BUCHANAN, J. M.; and DELLUVA, A. M. Biological Precursors of Uric Acid. I. The Role of Lactate, Acetate, and Formate in the Synthesis of the Ureide Groups of Uric Acid. J. Biol. Chem., **173**:69–79, 1948.
50. TOTTER, J. R.; VOLKIN, E.; and CARTER, C. E. The Distribution of Isotopically Labeled Formate in the Nucleotides of Ribo- and Desoxyribonucleic Acids. J. Am. Chem. Soc., **73**:1521–22, 1951.
51. WEYGAND, F.; WACKER, A.; and DELLWEG, H. Stoffwechseluntersuchungen bei Mikroorganismen mit Hilfe radioaktiver Isotope II. Kompetitive und nichtkompetitive Enthemmung von 5-^{82}Br-Uracil. Ztschr. Naturforsch., **7b**:19–25, 1952.
52. WHEELER, G. P., and SKIPPER, H. E. Chromatographic Evidence for Incorporation of 2,6-Diaminopurine into Nucleoside Phosphates of the Mouse. Fed. Proc., **12**:289, 1953.

Studies with Radioactive Colchicine

I. The Influence of Tumors on the Tissue Distribution of Radioactive Colchicine in Mice*

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In spite of the many papers published on colchicine and its influence on cell division, an acceptable explanation for the mode of action of this drug is not yet available. There is also still much uncertainty about the action of colchicine and its derivatives on tumors.

Ludford (13), using tumors and tissue culture, suggests that colchicine increases the viscosity of cell protoplasm. Northen (14), with onion root tips, found a decrease in the viscosity of protoplasm. Lettré (10) explains the action of colchicine on dividing cells by its interference with the regeneration of ATP from creatine phosphate. It is probable that the mode of action of colchicine should be sought in changes produced in one or several enzymatic complexes of the cell. Boyland and Boyland (3) reported changes in metabolism and ascorbic acid content after the injection of colchicine. Bloch-Frankenthal and Back (2) found that the alkaline phosphatase is reduced in a rat tumor but not in normal tissues of the same animal. Hawkins and Walker (9) found that when colchicine was given to hepatectomized rats it had no effect on the total amine oxidase activity of the liver but reduced the DOPA decarboxylase to about 50 per cent.

The contradictory results obtained by treating different tumors with colchicine can be summarized by saying that it seems that the growth of some tumors can be stopped, and the neoplasm may even be brought to complete regression if treatment is started in the earlier stages. Other tumors can be arrested only temporarily in their development, while still others can acquire a resistance to colchicine, as was recently reported by Lettré (10).

One of the difficulties in explaining the action of colchicine has been the impossibility of obtaining exact data concerning the distribution of colchicine in organs, tissues, and cells. The indirect methods of Brues (5) and Lettré (12), using regenerating rat liver and tissue culture, could give only comparative and qualitative indications of the presence of colchicine. The understanding of the correct mechanism of action of colchicine was further complicated by the fact that most of the biological work was done with colchicine which was contaminated with other colchicine-like alkaloids. Recently, Santavy (15) isolated from *Colchicum autumnale* no less than ten alkaloids, all of them with different toxicity and with different "mitotic poison" indices.

With the use of the biosynthetic technic (7), it has become possible to label with radioactive carbon (C^{14}) such complex drugs as colchicine, digitoxin, and morphine, which cannot be synthesized in the chemical laboratory.

In a preliminary paper (1) we described the distribution of pure radioactive colchicine in normal and in Sarcoma 180-bearing mice. In this communication we present the results of the distribution of colchicine in three other strains of tumor-bearing mice.

MATERIALS AND METHODS

Colchicine.—Pure radioactive colchicine labeled with carbon-14 was prepared by the biosynthetic procedures developed in this laboratory. Since this technic was described in a previous publication (17), only a brief statement of the procedure is necessary. Actively growing *Colchicum autumnale* plants were inclosed in air-tight chambers, into which C^{14} was introduced in a concentration of 50 μc/100 ml of carbon dioxide. The plants assimilated the radioactive carbon and incorporated it into all the constituents of the plant, one of

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which is the alkaloid colchicine. At the end of their growing period the plants were harvested, the corms dried, powdered, and extracted for their colchicine. The colchicine was purified by chromatography and identified by its physical constants

mp. 155–157°

$[\alpha]_D^{25} = -121.6°$ (in chloroform)

The specific activity of the colchicine so prepared varied from 7 to 25 μc/gm, depending on the assimilation of the radioactive carbon dioxide by the plants.

Mice and Tumors.—The mice used for these experiments were (*a*) C57BL, (*b*) BAF-1, cross C57BL females and A Jax males, (*c*) F_1 hybrids of DBA males and C3H females, and (*d*) white CF1. All animals were maintained on a Purina Dog Chow diet and were given free access to water. The tumors used were Sarcoma 180 on C57 mice, spindle-cell sarcoma on BAF-1, spontaneous mammary carcinoma on F_1 female hybrids of DBA and C3H. The Ehrlich ascites carcinoma was carried in white CF1. The two solid tumors were transferred by implantation in the scapular region and the ascites tumor by an intraperitoneal injection of approximately 50 million ascites cells.

After the tumors were 2–3 cm. in diameter or, in the case of Ehrlich ascites tumor after an interval of 7–10 days, radioactive colchicine was injected. For the mice bearing solid tumors, 1.0 mg. of colchicine in 0.5 cc. of distilled water was injected subcutaneously into the side opposite to the tumor; the same doses were injected intraperitoneally into mice bearing ascites tumors.

All animals were sacrificed 4 hours after injection. The organs were removed, dried between filter paper, weighed, and homogenized in distilled water. The homogenates were extracted with 70 per cent ethanol and filtered, and the filter paper was washed with additional 70 per cent ethanol. The filtrates were then evaporated to a small volume, diluted with water, and extracted with two 50-cc. portions of chloroform. The chloroform extract was evaporated to dryness, taken up in chloroform (C.P., 0.7 per cent ethyl alcohol) and passed through an alumina column. The resulting eluate contained colchicine, while other substances were adsorbed on the column. Equal parts of each sample were then analyzed with the use of an internal Geiger counter. The colchicine so isolated from the tissues was then identified by its characteristic ultraviolet absorption curve and by paper chromatography.

Essentially the same procedure was applied to the ascites tumor. The ascitic fluid with the free-swimming cells was removed with a syringe from the peritoneal cavity and centrifuged to separate the cells from the fluid. The cells were washed twice and then extracted by the method described above. A sample was taken before centrifugation to count the number of cells, with a blood counting chamber.

The lyophilized tissue injected into mice was prepared by the following method: The tumor tissue was removed from the mice, freed from necrotic tissue, and cut into small pieces. Throughout this operation the tissue was kept at 0° C. The small pieces of tissue were then dried in a freeze-drying apparatus with phosphorous pentoxide as desiccant, an ethylene glycol-dry ice mixture for lowering the temperature, and a high vacuum pump for evacuating the system. When the tissues were dry they were removed, powdered in a mortar, and stored in a refrigerator until used. The dry lyophilized tumor was suspended in saline and injected in doses of 80 mg/mouse; the controls were injected with 120 mg. of dry lyophilized muscle.

RESULTS

In our earlier paper (1) we reported that in normal C57 mice and in those bearing Sarcoma 180, some organs, e.g., heart, brain, blood, muscle, do not accumulate colchicine. However, in other organs, colchicine was present. In Table 1 we have summarized the distribution of colchicine in normal mice of three different strains. It can be seen that in all of them the spleen is the organ that accumulates the most colchicine. The intestine, kidney, and liver showed different amounts of accumulation of the drug. We believe this pattern can be accepted as a basis for the distribution of this drug in normal mice. In our tables the value of zero (0 per cent) indicates a counting rate less than 6 counts/minute above background. The backgrounds in the internal Geiger counters used were from 23 to 35 counts per minute, and the variations seen with any one counter were less than 4 counts per minute.

TABLE 1

DISTRIBUTION OF RADIOACTIVE COLCHICINE IN ORGANS OF THREE DIFFERENT STRAINS OF NORMAL MICE

	Intestine	Kidney	Liver	Spleen	Heart	Brain	Muscle
			(per cent)				
C57BL	22.8	28.6	9.4	39.2	0	0	0
BAF-1	19.2	22.6	15.1	43.1	0	0	0
CF-1 (white)	18.3	26.2	7.4	47.1	0	0	0

The counts found in each organ are expressed as a percentage of the total counts recovered. The percentage of administered dose recovered as unchanged colchicine was 4.4 per cent. Each value represents an average of at least ten animals.

Table 2 contains data on the distribution of labeled colchicine in animals bearing four different strains of tumors. In all four cases the distribution is similar, but different from the pattern of distribution in normal mice. There is a significant increase in the concentration of colchicine in the intestine; there is a slight rise of the concentration in the liver; and practically no change in the kidney. Each type of tumor also has a significant amount of colchicine. Most noteworthy is the change in the behavior of the spleen: In no tumor-bearing mouse of the four strains studied have we found a detectable amount of colchicine in the spleen.

To verify the role of the tumor in connection with the distribution of colchicine, we performed some control experiments. We have already reported that the injection of India ink to block the reticuloendothelial system was followed by normal behavior of the spleen. In a series of experiments, colchicine was injected into pregnant female mice in the second half of pregnancy. We presumed that the growth processes of tumor and of pregnancy might provoke identical reactions. However, the results indicated that the spleens of pregnant mice had the same colchicine content as did those of

normal mice. In the third series of experiments we inoculated each mouse with a tumor (C57/Sarcoma 180), allowed the tumor to grow to 2–2.5 cm. in diameter, and removed it surgically. When the wound had healed and the scar had attained a satisfactory appearance (7–9 days), the mice were given injections of radioactive colchicine. In all three series of experiments the spleen reacted normally, i.e., contained a large amount of colchicine. The results are shown in Table 3.

control, a normal mouse was given an injection of 120 mg. of lyophilized muscle from a normal mouse. The results of a few representative experiments are given in Table 4. It was found that lyophilized tumor produces a similar action to that of a tumor *in situ* and that lyophilized muscle introduced into the animal is inactive.

DISCUSSION

The understanding of tumor-host relations has been, and still is, the object of intensive research.

TABLE 2

DISTRIBUTION OF RADIOACTIVE COLCHICINE IN ORGANS OF TUMOR-BEARING MICE

Mice	Tumor	Intestine	Kidney	Liver (per cent)	Spleen	Tumor	Per cent of administered dose recovered
C57BL	Sarcoma 180	46.2	28.5	17.6	0	5.7	4.7
BAF-1	Spindle-cell sarcoma	39.5	28.1	19.0	0	13.4	4.1
DBA×C3H	Mammary carcinoma	38.2	30.7	18.5	0	12.3	4.7
CF-1 white	Ehrlich ascites carcinoma	39.8	26.7	26.7	0	6.8	

The counts found in each organ are expressed as a percentage of the total counts recovered. Each value represents an average of at least eight animals.

TABLE 3

DISTRIBUTION OF RADIOACTIVE COLCHICINE IN ORGANS OF PREGNANT, OPERATED UPON, AND NORMAL MICE

The counts found in each organ are expressed as a percentage of the total counts recovered. Each value represents an average of at least eight animals.

	Intestine	Kidney	Liver (per cent)	Spleen
Pregnant*	34.1	18.0	11.7	36.2
Operated upon†	34.7	23.4	7.2	34.7
Normal	22.7	28.5	9.2	39.1

* The mice were in the second half of pregnancy.

† The mice were used after the scars from the excision of the tumors had been closed and dry for 4–5 days (total time after operation, 7–9 days).

TABLE 4

DISTRIBUTION OF RADIOACTIVE COLCHICINE IN NORMAL MICE INJECTED WITH LYOPHILIZED TUMOR AND MUSCLE HOMOGENATES

	No.	Intestine	Kidney	Liver (per cent)	Spleen
Sarcoma 180	1	80.0	10.6	9.4	0
	2	29.5	54.1	16.4	0
	3	75.0	15.4	9.6	0
	4	88.3	0	11.7	0
	5	88.8	0	11.2	0
	6	58.5	25.8	15.4	0
Muscle	1	7.5	13.5	3.0	76.0
	2	10.5	0	3.3	86.0
	3	41.4	6.3	5.2	47.1
	4	20.2	0	29.2	50.6

The counts found in each organ are expressed as a percentage of the total counts recovered.

To verify the role played by the tumor in the behavior of the spleen, 80 mg. of lyophilized Sarcoma 180 was injected into normal mice. As a

With the introduction of the use of labeled substances a new approach to existing problems has been made possible.

Differences between the physiology of normal and tumor-bearing animals have been extensively described by Greenstein (8) and others (1, 2). Recently, in the work of Scott and Stone (16), alteration of the uptake of labeled iodotyrosine by thyroid, skin, blood, and gastrointestinal tract in tumor-bearing rats was described. Cerecedo and co-workers (6) reported that in sarcoma-bearing mice there was an increase in the arginine, histidine, cysteine, and threonine content of the livers; in lungs they observed a decrease in the histidine content. They did not observe any differences in the amino acid content of the spleens of tumor-bearing mice.

We believe that the facts reported by us support the previous findings of Scott and Stone (16) regarding the distribution of some radioactive substances in the intestines of tumor-bearing mice. Apparently, the intestine reacts to the presence of a tumor by a change in its physiology. This is the only apparent reason for the accumulation of iodotyrosine, colchicine, and chlorophyll[1, 2] in the intestine—substances different in both composition and physiological activity. This may partially explain the findings of Boyland (3) and Brues (4), that tumor-bearing animals are more sensitive to

[1] Unpublished data from our Laboratory indicate that tumor-bearing mice have 6–8 times more chlorophyll in the gastrointestinal tract than do normal mice.

[2] E. J. Walaszek, J. J. Kocsis, and A. Back, unpublished data.

colchicine than are the normal ones. It should be borne in mind that the dose of colchicine used in our experiments was a lethal one. However, it is known (4) that, once the lethal dose is reached, the size of that dose does not materially influence the interval before death of the animal.

The fact that the spleen of a tumor-bearing mouse does not accumulate colchicine may possibly be explained by the operation of a different mechanism. Is this reaction specific to colchicine or is it an expression of an important change produced by tumors? This point is now under investigation by us. It also has to be ascertained what part of the tumor causes this action. Further results will be reported by us in a forthcoming publication.

SUMMARY

1. The distribution of radioactive colchicine, prepared by biosynthesis, was studied in normal and tumor-bearing mice.

2. The spleens of normal mice accumulated significant amounts of injected radioactive colchicine (39–47 per cent of that recovered in the body), while none was found in the spleens of tumor-bearing mice. The intestines of tumor-bearing mice, however, accumulated *more* colchicine than the intestines of the normal mice.

3. When lyophilized tumor tissue was injected into normal mice, it had an action similar to that of a tumor *in situ*. It is postulated that a substance or substances present in tumor tissue provoke the described effect in spleen and intestine.

REFERENCES

1. BACK, A.; WALASZEK, E. J.; and UYEKI, E. Distribution of Colchicine in Some Organs of Normal and Tumor-bearing Mice. Proc. Soc. Exper. Biol. & Med., **77**:667–69, 1951.
2. BLOCH-FRANKENTHAL, L., and BACK, A. Effect of Colchicine on Tumor Growth and Tumor Pyrophosphatase. Proc. Soc. Exper. Biol. & Med., **76**:105–8, 1951.
3. BOYLAND, E., and BOYLAND, M. Studies in Tissue Metabolism. Action of Colchicine and *B. Typhosus* Extract. Biochem. J., **31**:454–60, 1937.
4. BRUES, A. M. Discussion Following a paper by M. Levine. Ann. N.Y. Acad. Sc., **51**:1407–9, 1951.
5. BRUES, A. M., and COHEN, A. Effects of Colchicine and Related Substances on Cell Division. Biochem. J., **30**: 1363–68, 1936.
6. CERECEDO, L.; REDDY, D.; LOMBARDO, M.; MCCARTHY, B.; and TRAVERS, J. J. Chemical Changes in Tumor-bearing Mice. Proc. Soc. Exper. Biol. & Med., **80**:723–26, 1952.
7. GEILING, E. M. K.; KELSEY, F. E.; MCINTOSH, B. J.; and GANZ, A. Biosynthesis of Radioactive Drugs Using Carbon 14. Science, **108**:558–59, 1948.
8. GREENSTEIN, J. Biochemistry of Cancer. New York: Academic Press, Inc., 1947.
9. HAWKINS, J., and WALKER, J. The Effect of Colchicine on the Enzyme Content of Regenerating Rat Liver. Brit. J. Pharmacol., **7**:152–60, 1952.
10. LETTRÉ, H. Über Mitosengifte. Erg. Physiol., **46**:379–452, 1950.
11. ———. Eine Gegen Colchicine Resistente Abart d. Mause-Ascites Tumor. Naturwissenschaften, **39**:266, 1952.
12. LETTRÉ, H., and LUTZE, M. Beitrag zur Verteilung u. Wirkung d. Colchicins in Tiercorpers. Hoppe-Seylers Ztschr. f. physiol. Chem., **281**:58–64, 1944.
13. LUDFORD, R. Colchicine in Experimental Chemotherapy. J. Nat. Cancer Inst., **6**:89–102, 1945.
14. NORTHEN, H. Alterations in the Structural Viscosity of Protoplasm by Colchicine and Their Relation to C-Mitosis and C-Tumor Formation. Am. J. Botany, **37**:705–11, 1950.
15. SANTAVY, F. Isolierung neuer Stoffe aus dem Knollen der Herbstzeitlosen, Colchicum autumnale L. Pharm. Acta Helv., **25**:248–65, 1950.
16. SCOTT, K. G., and STONE, R. S. Tumor Host Studies. II. Increased Concentration of Tagged Iodotyrosines in the Gastrointestinal Tract of Rats Bearing Tumors. Cancer, **3**:722–24, 1950.
17. WALASZEK, E. J.; KELSEY, F. E.; and GEILING, E. M. K. Biosynthesis and Isolation of Radioactive Colchicine. Science. **116**:225–37, 1952.

Systemic Effects of Tumors in Force-fed Rats

II. Effect on the Weight of Carcass, Adrenals, Thymus, Liver, and Spleen*†

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Progressive growth of the Walker 256 carcinoma produces anorexia and loss of carcass weight (6). Carcass weight loss has been prevented by force-feeding a high fat diet, but other systemic effects (decreased liver catalase activity, anemia, and adrenal enlargement) still occurred (1). It was suggested that loss of carcass weight is not a necessary component of the reactions leading to the development of systemic effects, and it was implied that the tumor's action as a nitrogen trap was not responsible for the production of such effects.

These experiments on force-fed, tumor-bearing rats have been repeated and extended to include high fat, high carbohydrate, and high protein diets. Data have been obtained also from rats fed a high fat diet and a chow diet ad libitum.

MATERIALS AND METHODS

Male (Sprague-Dawley) rats were used throughout. Several experiments were performed in which tumor-bearing and control rats were compared. The essential difference in each of these experiments was the composition of the diet.

In the original experiment (Ingle high fat diet), two groups of rats ranging in weight from 163 gm. to 200 gm. were selected by pairing, and the Walker 256 carcinoma was transplanted bilaterally and subcutaneously in the lumbar region of one group. Both groups were force-fed by stomach tube twice daily, beginning the day after grafting. Tap water was supplied ad libitum. The force-feeding was continued until the tumor had attained a size of over 20 per cent of the body weight, as calculated by Schrek's formula (9) from tumor measurements. The rats were then sacrificed following an overnight fast.

The method in subsequent experiments differed from the above as follows: The rats were selected by grouping according to body weight following preliminary observation of their growth rate. Force-feeding was begun 6 days after grafting, just prior to the first detectable growth of the tumor. The rats were sacrificed on the 21st day after transplantation of the tumor.

At the termination of the experiment, blood samples were drawn from the abdominal aorta under nembutal anesthesia. Heparin was used as an anticoagulant. This bleeding killed the rats.

The liver was removed, washed in water, blotted between paper towels, cleaned of adhering connective tissue, and weighed. It was then divided into 3 parts for water, nitrogen, and lipid analyses. The spleen, thymus, and adrenals were removed to moist paper, dissected free of adhering tissue, weighed, and replaced in the carcass. The gastrointestinal tract was removed from cardia to anus, slit open, washed with water, and replaced in the carcass. The tumors were removed and weighed. Tissues for nitrogen and lipid analyses (to be reported) were dissolved in 30 per cent alcoholic potassium hydroxide solution.

The diets used were: (*a*) the original Ingle high fat diet (4), (*b*) a synthetic high fat diet, similar to the Ingle diet in caloric composition (83 per cent of the calories were contributed by fat), and (*c*, *d*, and *e*) three synthetic diets containing the same ingredients as the synthetic high fat diet, but in which the major food constituent (fat, carbohydrate, or protein) contributed 60 per cent of the calories, and each of the other two contributed 20 per cent each. The composition of the diets is given in Table 1.

None of these diets was completely soluble in the liquid it contained. Prior to use they were mixed in a Waring Blendor and warmed to 30° C. Twenty-six ml. (67 calories) of the Ingle high fat diet and 40 ml. (70 calories) of the other diets were force-fed daily in two equal portions. In two experiments the rats were fed ad libitum either the maintenance fox chow diet[1] or the synthetic 60 per cent fat diet minus its water content.

RESULTS

The data are presented in Tables 2 and 3 according to the diet used. The results shown are mean values ± the standard error of the mean. Mean values have been compared by the *t* test of Fisher (10); values of P are regarded as significant at the 0.05 level.

The initial body weights were similar for all experiments. The final body weights of the force-

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[1] Master Fox Cubes, Toronto Elevators Limited.

fed controls were similar in all experiments in which the synthetic diets were used. Likewise, the final body weights, the carcass weights, and the tumor sizes of the tumor-bearing rats force-fed the synthetic diets were similar. It may be concluded that variation of the dietary composition over a considerable range had little or no influence on the growth of the rat bearing the Walker tumor, or of the tumor when isocaloric amounts of a nutritionally adequate diet were fed.

The final body weight, the carcass weight, and the tumor size of the rats force-fed the Ingle high fat diet are larger than for those fed the synthetic high fat diet. This is attributed to a 5-day longer growing period for the experiment employing the Ingle diet. The period of tumor growth was shortened in the experiments in which the rats were fed the synthetic diets in order to avoid the immediate pre-mortem period of tumor growth.

The final body weights of the force-fed tumor-bearing rats were always greater than the controls, although the food intakes of the two groups were identical. The tumor-bearing rats fed ad libitum also gained more weight per calorie of food in-

TABLE 1

COMPOSITION OF DIETS

	Ingle high fat	Synthetic high fat	Synthetic 60 per cent fat*	Synthetic 60 per cent carbohydrate*	Synthetic 60 per cent protein*
Egg albumen (gm.)	18.2	8.1	8.2	6.3	18.8
Lactalbumen (gm.)		18.9	19.1	14.6	43.9
Corn oil (gm.)	22.7	58.4	33.9	7.4	7.4
Butter (gm.)	29.5				
Sucrose (gm.)		0.4	27.2	62.7	20.9
Dried yeast (gm.)	11.4				
Salt mixture (gm.)	4.5†	6.6‡	5.4‡	4.2‡	4.2‡
Cellu flour (gm.)	13.6	7.6	6.2	4.8	4.8
Total	99.9	100.0	100.0	100.0	100.0
Water-soluble vitamin mixture ml/100 gm of above§		3.33	2.74	2.10	2.10
Fat-soluble vitamin mixture ml/100 gm of above#	2.0	3.33	2.74	2.10	2.10
Water, 100 gm. diet to	227.3 ml.	378.4 ml.	311.1 ml.	238.9 ml.	238.9 ml.

* The per cent refers to the percentage caloric contribution of the major constituent of the diet.

† Hawk-Oser salt mixture (2).

‡ Salt mixture of Phillips and Hart (7) modified to contain 75.2 mg cobalt/kg of salt mixture.

§ Water-soluble vitamin mixture: thiamine, 50 mg.; pyridoxine, 50 mg.; riboflavin, 100 mg.; nicotinic acid, 500 mg.; calcium pantothenate, 590 mg.; biotin, 2 mg.; folic acid, 5 mg.; inositol, 1 gm.; choline, 10 gm.; 95 per cent ethanol, 50 ml.; water, to 100 ml.

Fat-soluble vitamin mixture: cod liver oil, 5 gm.; wheat germ oil, 5 gm.; and 2-methyl-naphthoquinone, 50 mg.

TABLE 2

THE EFFECT OF THE WALKER 256 CARCINOMA ON BODY AND CARCASS WEIGHT OF RATS FED VARIOUS DIETS

Diet	Rat	Initial body wt. (gm.)	Final body wt. (gm.)	Carcass wt.* (gm.)	Tumor size† (per cent)
Force-fed:					
Ingle high fat	Tumor (7)	179±4.0	314± 5.4‡	244± 3.7§	22.4±1.3
	Control (7)	180±4.4	255± 3.1	255± 3.1	
Synthetic high fat	Tumor (8)	177±2.5	280± 4.2‡	229± 3.1‡	18.1±1.3
	Control (8)	177±2.3	242± 2.7	242± 2.7	
Synthetic 60 per cent fat#	Tumor (8)	176±3.1	293± 8.1‡	235± 3.7	19.3±1.9
	Control (8)	175±1.8	241± 2.8	241± 2.8	
Synthetic 60 per cent carbohydrate#	Tumor (8)	174±1.0	287± 5.1‡	232± 6.5§	19.0±2.1
	Control (8)	177±2.0	248± 1.8	248± 1.8	
Synthetic 60 per cent protein#	Tumor (8)	181±1.8	284±16.4§	225± 8.7	20.4±1.6
	Control (8)	181±1.8	237± 3.2	237± 3.2	
Ad libitum:					
Synthetic 60 per cent fat	Tumor (6)	168±2.0	255±13.7	198±10.6‡	23.1±2.8
	Control (6)	169±4.1	270± 6.5	270± 6.5	
Chow	Tumor (9)	176±3.1	255± 9.3	193± 7.8‡	24.1±0.9
	Control (9)	175±1.8	255±10.5	255±10.5	

* Carcass weight = body weight − tumor weight.

† Tumor size is expressed as per cent of final body weight.

‡ $P < 0.01$

§ $P < 0.05$

The per cent refers to the percentage caloric contribution of the major constituent of the diet.

gested than did their controls, although in this case the difference between the two groups is due to a decreased food intake on the part of the tumor-bearers[2] rather than to a difference in weight gain. Mider *et al.* (6) obtained similar results by comparing the growth of tumor-bearing rats, feeding ad libitum, to pair-fed control rats.

The carcass weight of the force-fed tumor-bearers was significantly less than the controls on the Ingle high fat diet, the synthetic 83 per cent fat, and the synthetic 60 per cent carbohydrate diets; on the other two diets it is less, but not significantly so. Thus, in contrast to the earlier observations (1), maintenance of the food intake by force-feeding did not permit complete maintenance of the carcass weight of the rat bearing the Walker tumor when the tumor was permitted to grow to 20 per cent of the body weight. However, it did permit much more carcass growth than in tumor-bearing rats fed ad libitum.

The tumor appeared to have attained a greater size in rats fed ad libitum, in spite of the greater food intake of the force-fed rats. Presumably, the stress of force-feeding depressed tumor growth. In this connection it is of interest that cortisone (5) decreased the rate of growth of the Walker tumor in its early stages.

The organ weights presented in Table 3 show clearly that force-feeding does not prevent the enlargement of the adrenals, spleen, and liver or the involution of the thymus in the rats bearing the Walker tumor. The weight of the thymus appears to be smaller in rats fed the 60 per cent protein diet than in rats fed the other diets. Likewise the weights of the thymus were smaller in the force-fed rats than in those fed ad libitum. Conversely, the adrenals of the force-fed rats appeared to be larger than those of rats fed ad libitum, except in the group fed the 60 per cent fat diet ad libitum, where the extreme enlargement of the adrenals in one tumor-bearing rat[3] raised the mean of this group to that of the force-fed rats.

TABLE 3

THE EFFECT OF THE WALKER 256 CARCINOMA ON THE WEIGHT OF ADRENALS, THYMUS, LIVER, AND SPLEEN OF RATS FED VARIOUS DIETS*

Diet	Rat	Adrenals (mg.)	Thymus (mg.)	Spleen (gm.)	Liver (gm.)
Force-fed:					
Ingle high fat	Tumor (7)	79± 7.1	107±15	3.60±0.30	19.3±3.2
	Control (7)	41± 3.2	310±16	0.58±0.04	9.8±0.6
Synthetic high fat	Tumor (8)	74± 5.3	166±16	3.24±0.35	16.7±1.0
	Control (8)	39± 1.3	272±16	0.55±0.02	9.6±0.2
Synthetic 60 per cent fat†	Tumor (8)	73± 4.9	195±21	3.58±0.27	16.1±0.5
	Control (8)	53± 2.7	359±35	0.63±0.03	10.2±0.3
Synthetic 60 per cent carbohydrate†	Tumor (8)	73± 5.5	160±28	3.55±0.58	16.8±0.9
	Control (8)	53± 1.0	306±35	0.62±0.30	10.8±0.4
Synthetic 60 per cent protein†	Tumor (8)	86± 5.6	82±13	4.02±0.36	17.1±0.8
	Control (8)	55± 4.0	236±15	0.51±0.02	11.1±0.2
Ad libitum:					
Synthetic 60 per cent fat	Tumor (6)	62±13.8	243±59	3.47±0.62	15.7±0.7
	Control (6)	38± 1.0	469±29	0.79±0.06	11.8±0.7
Chow	Tumor (9)	64± 3.5	231±33		
	Control (9)	38± 1.5	480±39		

* $P < 0.01$ for all comparisons between tumor rats and their controls on each diet.
† The per cent refers to the percentage caloric contribution of the major constituent of the diet.

DISCUSSION

A considerable variability with large standard errors of the mean may be noted for most of the reported systemic effects. It appears that there are marked differences in the response of a few rats to the presence of the Walker tumor. This may explain the failure of Begg and Dickinson (1) to note any loss of carcass weight in four tumor-bearing rats force-fed the Ingle high fat diet.

The carcass weights reported in this paper include the contents of the gastrointestinal tract. Although the rats had been fasted overnight, it is possible that the amount of intestinal contents of the tumor-bearers may have differed from that of the controls, but this was not apparent on gross examination. A difference may also have existed between the amounts of the gastrointestinal contents in the force-fed rats and those fed ad libitum. The extent to which such differences may have affected the results is unknown.

It is also uncertain to what extent the level of food intake may have influenced the results. The

[2] The ad libitum intake of food over a 10-day period, beginning on the 10th day after the tumor graft, was 34.2 ± 3.6 calories per rat per day for the rats bearing tumors and 54.8 ± 1.9 calories per rat per day for the control rats. There were twelve rats in each group, and they were fed a diet in which fat furnished 60 per cent of the calories.

[3] The adrenals of this rat weighed 142 mg.

force-fed rats received 70 calories per day. This may be considered to be overfeeding when compared to the approximately 55 calories per day ingested by the nontumor-bearing rats fed ad libitum.[2]

The deficit in carcass weight of the tumor-bearing rat observed in these experiments may also be explained by the nitrogen trap concept of tumor action (6). Could the nitrogen trap also be responsible for the other effects? Maintenance of the food intake by force-feeding the tumor rat has prevented much of the carcass weight deficit; it has not resulted in a similar improvement in the observed systemic effects. It will be shown in paper III of this series that there is a deficit of carcass nitrogen[4] in the force-fed rat bearing the Walker tumor of a magnitude similar to the carcass weight deficit. This supports the nitrogen trap concept but provides no answer to the problem of its correlation with the production of other effects of the tumor.

The dietary modifications used in the force-feeding experiments have had comparatively little influence on the magnitude of most of the observed systemic effects. The changes which have been noted when the weights of the thymus and adrenal glands of the force-fed rats are compared to those fed ad libitum may be attributed to the stress of force-feeding mediated through the pituitary-adrenal system (8). This stimulation of the pituitary-adrenal system by force-feeding introduces another variable in addition to the food intake and makes difficult, if not impossible, comparison of the force-fed rats with those fed ad libitum. The low weights of the thymus of the rats fed the high protein diet may also be attributed to adrenal stimulation, although the accompanying adrenal enlargement was not statistically significant. Enlargement of the adrenals in rats fed high protein diets has been reported (3, 11).

SUMMARY

Force-feeding high fat, carbohydrate, and protein diets to rats bearing the Walker 256 carcinoma greatly reduced, but did not prevent, the loss of carcass weight noted in tumor-bearing rats fed ad libitum.

Maintenance of the food intake did not alter the enlargement of liver and spleen that occurs in tumor-bearing rats fed ad libitum, but some increase in adrenal enlargement and thymus involution was observed, presumably owing to the stress of force-feeding.

[4] The carcass nitrogen values were not influenced by the contents of the gastrointestinal tract, since these were removed by washing prior to analysis.

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REFERENCES

1. BEGG, R. W., and DICKINSON, T. E. Systemic Effects of Tumors in Force-fed Rats. Cancer Research, **11**:409–12, 1951.
2. HAWK, P. B.; OSER, B. L.; and SUMMERSON, W. H. Practical Physiological Chemistry, p. 1273. 12th ed. Philadelphia and Toronto: The Blakiston Company, 1947.
3. INGLE, D. J. A Further Study of the Effect of Diet on Adrenal Weights in Rats. Endocrinology, **37**:7–14, 1945.
4. INGLE, D. J.; PRESTRUD, M. C.; LI, C. H.; and EVANS, H. M. The Relationship of Diet to the Effect of Adrenocorticotrophic Hormone upon Urinary Nitrogen, Glucose, and Electrolytes. Endocrinology, **41**:170–76, 1947.
5. INGLE, D. J.; PRESTRUD, M. C.; and RICE, K. L. The Effect of Cortisone Acetate upon the Growth of the Walker Rat Carcinoma and upon Urinary Non-Protein Nitrogen, Sodium, Chloride and Potassium. Endocrinology, **46**:510–13, 1950.
6. MIDER, G. B.; TESLUK, H.; and MORTON, J. J. Effect of the Walker Carcinoma 256 on Food Intake, Body Weight and Nitrogen Metabolism of Growing Rats. Acta Union internat. contre cancer, **6**:409–20, 1948.
7. PHILLIPS, P. H., and HART, E. B. The Effect of Organic Dietary Constituents upon Chronic Fluorine Toxicosis in the Rat. J. Biol. Chem., **109**:657–63, 1935.
8. SAYERS, G., and SAYERS, M. A. The Pituitary-Adrenal System. Recent Progress in Hormone Research, **2**:81–115, 1948.
9. SCHREK, R. A Quantitative Study of the Growth of the Walker Rat Tumor and Flexner-Jobling Rat Carcinoma. Am. J. Cancer, **24**:807–22, 1935.
10. SNEDECOR, G. W. Statistical Methods, p. 75. 4th ed. Ames: Iowa State College Press, 1946.
11. TEPPERMAN, J.; ENGEL, F. L.; and LONG, C. N. H. Effect of High Protein Diets on Size and Activity of the Adrenal Cortex in the Albino Rat. Endocrinology, **32**:403–9, 1943.

Systemic Effects of Tumors in Force-fed Rats

III. Effect on the Composition of the Carcass and Liver and on the Plasma Lipids*†

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The force-feeding of rats bearing the Walker 256 carcinoma largely prevented the marked loss of carcass weight that occurs in the tumor-bearing rat fed ad libitum but had little influence on the magnitude of several other systemic effects (2, 21). It was suggested that the action of the tumor as a nitrogen trap was unlikely to be a necessary component of the reaction leading to the development of systemic effects (2). Carcass nitrogen analyses were not done, so that it was unknown whether or not nitrogen loss had occurred. In this paper the results of the carcass analyses of the same force-fed rats that were reported in the preceding paper of this series are presented (21).

It was also observed in the earlier work that rats bearing the Walker tumor and force-fed a high fat diet developed a hyperlipemia which prevented accurate estimation of hemoglobin by the usual photocolorimetric methods (2). The lipid content of the plasma of these rats was not measured. These rats also had fatty livers. Hyperlipemia (6) and marked loss of carcass lipid (14) in tumor-bearing rats fed ad libitum have been reported. It was of interest to determine the lipid content of the carcass, liver, and blood plasma of force-fed, tumor-bearing rats.

MATERIALS AND METHODS

The rats used in these experiments were the same as those described in the previous paper (21) in which the method of autopsy and the composition of the diets are given.

The nitrogen contents of carcass, tumor, and liver were determined on aliquots of the alcoholic KOH solutions of the tissues by a semimicro-Kjeldahl method (17) with steam distillation into 2 per cent boric acid solution and a mixed indicator (11). The approximate amounts of tissue and the final volumes of their solutions were: for carcass, 200 gm. in 500 ml.; tumor, 60 gm. in 200 ml.; and liver, 10 gm. in 50 ml.

The fatty acid analyses of the carcass and tumor were performed on 10-ml. aliquots of the same tissue solutions used for the nitrogen analyses, according to the method of Haven, Bloor, and Randall (5).

The total lipids of blood plasma and liver were determined by weighing the material extracted with petroleum ether from the residue remaining after removal of the solvent from aliquots of alcohol-ether extracts of the tissues. The extractions were carried out according to Bloor (3).

The blood and liver phospholipids were calculated from phosphorus analyses of aliquots of the alcohol-ether extracts of these tissues according to the method of Fiske and Subba-Row (4). The factor 22.7 of Artom and Fishman (1) was used to convert the phosphorus values to phospholipid.

Blood total cholesterol was determined by the method of Sperry (20), with larger volumes as described in Hawk, Oser, and Summerson (7). Mean values have been compared by the *t* test of Fisher (19); values of P are regarded as significant at the 0.05 level.

RESULTS

The results shown are mean values ± the standard error of the mean. It is evident from Table 1 that force-feeding failed to prevent the loss of nitrogen from the carcass of the tumor-bearing rat, though the losses were not so great as those in the tumor-bearing rat fed ad libitum. This is in accord with the values for the gross carcass weight of these rats (21).

The total body nitrogen of the force-fed tumor-bearers was greater than their corresponding controls in all cases except those on the 60 per cent protein diet, but in no instance was the difference statistically significant. At the same time, the force-fed tumor rat had a considerably greater total body weight than the nontumor-bearing rat (21). Calculation based on a tumor water content of 83 per cent[1] suggests that water accounted for a

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[1] The mean value for nine large tumors was 83.1 ± 0.2 per cent.

large part of the increased weight of the tumor-bearer.

The fatty acid content of the carcasses of the tumor-bearers shown in Table 2 was less than that of their controls. The data in this table are incomplete owing to a technical error. The data which are shown for the synthetic high fat and synthetic 60 per cent diets[2] were obtained in preliminary studies of the dietary modifications. These rats were not selected so rigidly, were approximately 30 gm. larger, and bore tumors approximately 24 per cent of the body weight. Again it may be said that force-feeding failed to prevent losses of carcass fatty acid but that the losses were not so great as those in tumor-bearing rats fed ad libitum.

The total fatty acid content of the tumor-bearing rat, whether force-fed or fed ad libitum, was less than that of the control rat. On the synthetic 60 per cent carbohydrate and 60 per cent protein diets this difference was statistically significant in spite of the fact that the total body weights of the tumor-bearers were greater than the controls. This is not surprising in view of the decreased fatty content of the carcass and the low lipid content of

[2] The diets have been named according to the percentage caloric composition of their major constituent (fat, carbohydrate, or protein). Fat contributed 83 per cent of the calories in the high fat diets.

TABLE 1

THE EFFECT OF WALKER 256 CARCINOMA ON THE DISTRIBUTION OF NITROGEN IN RATS FED VARIOUS DIETS

Diet	Rat	Carcass nitrogen (gm.)	Tumor nitrogen (gm.)	Total nitrogen (gm.)
Force-fed:				
Ingle high fat	Tumor (7)	5.99±0.19	1.20±0.07	7.19±0.24
	Control (7)	6.67±0.26		6.67±0.26
Synthetic high fat	Tumor (8)	5.47±0.10*	0.88±0.04	6.34±0.08
	Control (8)	6.03±0.15		6.03±0.15
Synthetic 60 per cent fat†	Tumor (8)	5.53±0.21	0.88±0.07	6.28±0.18
	Control (8)	6.00±0.21		6.00±0.21
Synthetic 60 per cent carbohydrate†	Tumor (8)	6.08±0.23*	0.98±0.08	7.06±0.17
	Control (8)	6.85±0.10		6.85±0.10
Synthetic 60 per cent protein†	Tumor (8)	5.52±0.18*	0.99±0.10	6.51±0.24
	Control (8)	6.62±0.11		6.62±0.11
Ad libitum:				
Synthetic 60 per cent fat	Tumor (6)	5.09±0.32*	0.89±0.12	5.98±0.30‡
	Control (6)	7.05±0.17		7.05±0.17
Chow	Tumor (4)	5.62±0.41*	1.05±0.05	6.68±0.43‡
	Control (4)	7.86±0.29		7.86±0.29

* $P < 0.01$.
† The per cent refers to the percentage caloric contribution of the major constituent of the diet.
‡ $P < 0.05$.

TABLE 2

THE EFFECT OF WALKER 256 CARCINOMA ON THE DISTRIBUTION OF FATTY ACIDS IN RATS FED VARIOUS DIETS

Diet	Rat	Carcass fatty acids (gm.)	Tumor fatty acids (gm.)	Total fatty acids (gm.)
Force-fed:				
Ingle high fat	Tumor (7)	27.3±1.3	2.18±0.46	29.5±1.1
	Control (7)	30.3±2.4		30.3±2.4
Synthetic high fat	Tumor (2)	25.1	2.34	27.4
	Control (2)	29.5		29.5
Synthetic 60 per cent fat*	Tumor (3)	27.1	2.33	29.4
	Control (3)	34.2		34.2
Synthetic 60 per cent carbohydrate*	Tumor (8)	18.3±0.8†	1.28±0.14	19.7±0.8‡
	Control (8)	22.2±0.8		22.2±0.8
Synthetic 60 per cent protein*	Tumor (8)	15.7±1.2†	1.15±0.10	16.6±1.3†
	Control (8)	23.5±1.5		23.5±1.5
Ad libitum:				
Synthetic 60 per cent fat	Tumor (6)	11.2±2.4†	1.38±0.21	12.6±2.4†
	Control (6)	26.7±2.2		26.7±2.2
Chow	Tumor (4)	5.5±1.3†	1.08±0.10	6.5±1.3†
	Control (4)	20.5±1.9		20.5±1.9

* The per cent refers to the percentage caloric contribution of the major constituent of the diet.
† $P < 0.01$.
‡ $P < 0.05$.

the tumor tissue. It is of interest that the few results available suggest that the tumors of the rats on the high fat diets contained more lipid than those on the other diets.

The hyperlipemia which developed in the force-fed, tumor-bearing rat is seen from Table 3 to be very marked in those rats on the high fat diets. The highest value observed was over 15,000 mg. of total lipid/100 ml of plasma for a rat force-fed the synthetic high fat diet and bearing a tumor which was 19 per cent of the body weight. In gross appearance the plasmas of these rats with extreme hyperlipemia were indistinguishable from milk. It stituents made up only a small fraction of the total plasma lipid increase in those rats with extreme hyperlipemia. Presumably neutral fat was largely responsible for the increase.

The composition of the livers is shown in Table 4. Fatty livers were obtained only in the rats fed the Ingle high fat diet. The total lipid content of 49 per cent of the dry weights for the tumor-bearing rats on this diet corresponds to 17 per cent of the wet weight. Accompanying the increased lipid content of these livers was a decreased water content. The decrease in water content occurred in both tumor-bearers and controls and to about the

TABLE 3

THE EFFECT OF WALKER 256 CARCINOMA ON PLASMA LIPIDS OF RATS FED VARIOUS DIETS

Diet	Rat	Total lipid mg/100 ml	Phospholipid mg/100 ml	Cholesterol mg/100 ml
Force-fed:				
Ingle high fat	Tumor (7)	5598 ± 591*	435 ± 33*	175 ± 15.4*
	Control (7)	214 ± 8	103 ± 6	50 ± 2.5
Synthetic high fat	Tumor (8)	5916 ± 1659*	443 ± 67*	154 (3)†
	Control (8)	289 ± 17	111 ± 4	44 (3)†
Synthetic 60 per cent fat‡	Tumor (8)	1983 ± 539*	280 ± 33*	96 (2)†
	Control (8)	225 ± 14	83 ± 4	54 (2)†
Synthetic 60 per cent carbohydrate‡	Tumor (8)	1122 ± 232*	264 ± 35*	
	Control (8)	228 ± 25	112 ± 5	
Synthetic 60 per cent protein‡	Tumor (8)	1217 ± 327*	309 ± 43*	
	Control (8)	213 ± 13	131 ± 4	
Ad libitum:				
Synthetic 60 per cent fat	Tumor (6)	1616 ± 472§	251 ± 35*	
	Control (6)	248 ± 22	115 ± 3	

* $P < 0.01$.
† The numbers in brackets refer to the number of rats.
‡ The per cent refers to the percentage caloric contribution of the major constituent of the diet.
§ $P < 0.05$.

is not surprising that Begg and Dickinson (2) had difficulty in measuring blood hemoglobin in such rats.

The degree of hyperlipemia was very variable and appears to be correlated with the amount of fat in the diet. This is evident when the results for the rats force-fed the high fat diets are compared to those for the rats fed the diets containing 20 per cent fat (synthetic 60 per cent carbohydrate and 60 per cent protein diets). Comparison of the plasma total lipid value for the rats fed the 60 per cent fat diet ad libitum, with the median value of 325 mg/100 ml obtained by Haven *et al.* (5) for rats eating a chow diet and bearing Walker tumors, reveals the same picture. Milky plasmas are not so common in rats fed ad libitum but they do occur in rats on a high fat diet and bearing large tumors.

The plasma phospholipid and cholesterol values also showed a marked increase in force-fed tumor-bearers. The increases were not strictly proportional to the increases in total lipid. These con-

same extent. An increased water content is usually observed in the liver of the tumor-bearing rat (12). This lack of increase in the rats fed the Ingle high fat diet may be related to their high fat content.

When the experiments with the Ingle diet were performed, it was thought that the fatty livers observed in rats force-fed this diet might be attributed to a dietary choline deficiency. The absence of fatty livers in the rats fed the synthetic high fat diet which contained a synthetic water-soluble vitamin mixture in place of the yeast of the Ingle diet would seem to confirm this view. The water content of the livers of the tumor-bearing rats fed the synthetic diets showed the increased water content that was expected but not observed in the rats force-fed the Ingle diet.

The total lipid content of the livers of the tumor-bearing rats force-fed the synthetic 60 per cent carbohydrate and the synthetic 60 per cent protein diets was greater than that of their controls. This difference was not observed in the rats fed the other synthetic diets. The main difference

appears to be in the controls, for the total lipid content of the controls increased when the fat content of the diet was increased from 20 per cent to 60 per cent of the calories, whereas that of the tumor-bearers did not. When the fat content of the diet was further increased to 83 per cent of the calories, the lipid content of the livers of both tumor-bearing and control rats increased. The results suggest that the livers of normal rats may metabolize lipid more efficiently than tumor-bearers at the lower levels of fat intake but not at higher levels. This may be attributable to a difference between the tumor and nontumorous rats in their choline requirement.

The lower nitrogen content of the carcass of the force-fed rat bearing the Walker tumor, when compared to that of the control rat, indicates some protein depletion if the control is taken as the base-line. This protein depletion is more severe in the rat fed ad libitum and reflects the decreased food intake of these rats. It is what would be expected if the tumor functioned as a nitrogen trap. As the tumor enlarges, its demand for nitrogenous building blocks increases. When the demand exceeds the dietary supply, the carcass of the host makes up the difference. In the force-fed rat the point of equilibrium is reached later when the tumor is larger, since the food intake is greater;

TABLE 4

THE EFFECT OF WALKER 256 CARCINOMA ON THE COMPOSITION OF THE LIVER OF RATS FED VARIOUS DIETS

Diet	Rat	Water (per cent)*	Nitrogen (per cent)†	Total lipid (per cent)†	Phospholipid (per cent)†
Force-fed:					
Ingle high fat	Tumor (7)	64.6±1.9	6.96±0.51	49.0±2.8	10.1±0.8
	Control (7)	64.1±3.6	8.68±1.20	43.5±4.3	10.9±0.9
Synthetic high fat	Tumor (8)	72.9±0.6‡	8.23±0.20	26.9±0.9	12.9±0.3
	Control (8)	69.5±0.3	8.48±0.45	27.7±1.3	13.2±0.3
Synthetic 60 per cent fat§	Tumor (8)	70.7±0.4‡	7.88±0.21#	20.1±0.5	11.8±0.6
	Control (8)	67.6±1.5	7.11±0.22	20.5±2.3	10.9±0.7
Synthetic 60 per cent carbohydrate§	Tumor (8)	73.5±0.7‡	9.75±0.31	20.2±0.6‡	13.9±0.6
	Control (8)	71.6±0.4	10.19±0.84	16.7±0.5	13.8±0.5
Synthetic 60 per cent protein§	Tumor (8)	72.8±0.4‡	10.44±0.49	23.1±1.2‡	11.7±0.6
	Control (8)	69.0±0.3	10.01±0.23	18.4±0.4	11.6±0.6
Ad libitum:					
Synthetic 60 per cent fat	Tumor (6)	72.9±1.3	8.84±0.48	20.5±1.0	11.1±0.6
	Control (6)	71.3±0.6	8.20±0.41	23.3±0.8	10.9±0.2

* Based on wet weight.
† Based on dry weight.
‡ $P < 0.01$.
§ The per cent refers to the percentage caloric contribution of the major constituent of the diet.
# $P < 0.05$.

The phospholipid content of the livers does not suggest any disturbance of this aspect of lipid metabolism in the tumor-bearing rat, for it was the same in tumorous and control rats on all diets. Variation of the fat content of the diet had no consistent effect on the level of phospholipid in the liver. This reaffirms the relative constancy of this component under a variety of conditions.

DISCUSSION

The force-fed, tumor-bearing rat gains more weight than the noncancerous rat fed an isocaloric amount of food, but calculation suggests that much of the extra weight is due to water. The force-fed tumor-bearing rat contains slightly less nitrogen and fat than the control. This implies that the tumorous rat contains fewer calories in spite of his increased weight, which is in agreement with bomb calorimetry studies on pair-fed rats (13).

and the result is a less severe depletion of the force-fed rat. The data, therefore, support the concept of the tumor as a nitrogen trap.

The water and nitrogen analyses, in conjunction with the increase in liver weight (21), suggest a synthesis of new protein by the liver. This is noted also in the ad libitum high fat group where there was a considerable loss of carcass weight (21). At this stage of tumor growth the liver can compete successfully with the tumor for protein building blocks (16, 18).

The extreme hyperlipemia which develops in the tumor-bearing rats force-fed the high fat diets is startling. It is much greater than has been observed heretofore in cancerous rats, owing, undoubtedly, to the greater fat intake. Haven *et al.* (5) suggested originally that hyperlipemia in cancerous rats is due to mobilization of the fat stores in response to an increased caloric requirement of the host or to hormonal disturbance. They have

suggested more recently that it may be due to mobilization of the fat stores in response to the tumor's demand for unsaturated fatty acids needed for the formation of phospholipid and cholesterol esters in the tumor (6).

These hypotheses appear unconvincing as an explanation for the extreme hyperlipemia. It is difficult to envision the necessity for mobilizing more fat when the blood already contains large quantities of it. The combination of impaired entrance and unimpaired exit of lipid to and from the fat stores, together with continued absorption of dietary lipid, could account for hyperlipemia and depletion of the carcass lipids. It would be of interest to determine whether fat deposition can occur in a tissue in a state of protein catabolism.

The hyperlipemia might also be attributed to hyperfunction of the adrenal cortex. Cortisone (10) but not ACTH (8) produced visible hyperlipemia and fatty livers in rats. Preliminary investigation in this laboratory revealed no increase in plasma lipid content of rats treated with sufficient ACTH to prevent growth. Daily administration of 3 mg. of cortisone for 12 days to three rats raised the plasma total lipid to only 480 mg/100 ml. Attempts to prevent hyperlipemia by adrenalectomy of force-fed tumor-bearing rats have been unsuccessful owing to early deaths.

The composition of the livers is noteworthy for the similarity between the tumor-bearers and controls. These estimations were made in the first instance in anticipation of evidence indicating impaired lipid metabolism. The use of more adequate diets than the Ingle high fat diet fed originally suggests that if there is any such impairment the liver is not involved.

SUMMARY

The effects of the Walker 256 carcinoma have been studied in male rats force-fed high fat, carbohydrate, and protein diets until the tumor attained a size of approximately 20 per cent of the body weight.

The loss of nitrogen and fatty acids from the carcasses of tumor-bearing rats was diminished but not prevented by forced feeding.

The total lipid content of the plasma was variable and reached very high values in tumor-bearing rats fed high fat diets. Increases in phospholipid and total cholesterol accounted for only a small fraction of the increase, which is presumably owing to neutral fat.

The nitrogen, total lipid, and phospholipid contents of the livers of tumor-bearers were similar to those of control rats.

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REFERENCES

1. Artom, C., and Fishman, W. H. The Relation of the Diet to the Composition of Tissue Phospholipids. J. Biol. Chem., **148**:405–14, 1943.
2. Begg, R. W., and Dickinson, T. E. Systemic Effects of Tumors in Force-Fed Rats. Cancer Research, **11**:409–12, 1951.
3. Bloor, W. R. The Oxidative Determination of Phospholipid (Lecithin and Cephalin) in Blood and Tissues. J. Biol. Chem., **82**:273–86, 1929.
4. Fiske, C. H., and SubbaRow, Y. The Colorimetric Determination of Phosphorus. J. Biol. Chem., **66**:375–400, 1925.
5. Haven, F. L.; Bloor, W. R.; and Randall, C. Lipids of the Carcass, Blood Plasma and Adrenals of the Rat in Cancer. Cancer Research, **9**:511–14, 1949.
6. ———. The Nature of the Fatty Acids of Rats Growing Walker Carcinoma 256. *Ibid.*, **11**:619–23, 1951.
7. Hawk, P. B.; Oser, B. L.; and Summerson, W. H. Practical Physiological Chemistry, pp. 531–34, 12th ed. Philadelphia and Toronto: The Blakiston Company, 1947.
8. Ingle, D. J.; Prestrud, M. C.; and Li, C. H. Effect of Administering Adrenocorticotrophic Hormone by Continuous Injection to Normal Rats. Am. J. Physiol., **166**: 165–70, 1951.
9. Ingle, D. J.; Prestrud, M. C.; Li, C. H.; and Evans, H. M. The Relationship of Diet to the Effect of Adrenocorticotrophic Hormone upon Urinary Nitrogen, Glucose and Electrolytes. Endocrinology, **41**:170–76, 1947.
10. Ingle, D. J.; Prestrud, M. C.; and Nezamis, J. Effect of Administering Large Doses of Cortisone Acetate to Normal Rats. Am. J. Physiol., **166**:171–75, 1951.
11. Ma, T. S., and Zuazaga, G. Micro-Kjeldahl Determination of Nitrogen—A New indicator and Improved Rapid Method. Ind. & Eng. Chem., Anal. Ed., **14**:280–82, 1942.
12. McEwen, H. D., and Haven, F. L. The Effect of Carcinosarcoma 256 on the Water Content of the Liver. Cancer Research, **1**:148–50, 1941.
13. Mider, G. B.; Fenninger, L. D.; Haven, F. L.; and Morton, J. J. The Energy Expenditure of Rats Bearing Walker Carcinoma 256. Cancer Research, **11**:731–36, 1951.
14. Mider, G. B.; Sherman, C. D.; and Morton, J. J. The Effect of Walker 256 Carcinoma on the Total Lipid Content of Rats. Cancer Research, **9**:222–24, 1949.
15. Mider, G. B.; Tesluk, H.; and Morton, J. J. Effects of Walker Carcinoma 256 on Food Intake, Body Weight and Nitrogen Metabolism of Growing Rats. Acta Union internat. contre cancer, **6**:409–20, 1948.
16. Norberg, E., and Greenberg, D. M. Incorporation of Labeled Glycine in the Protein of Tissues of Normal and Tumor-Bearing Mice. Cancer, **4**:383–86, 1951.

17. PREGL, G. Quantitative Organic Microanalysis, p. 84, 4th English Ed. London: Churchill, 1945.
18. SHERMAN, C. D.; MORTON, J. J.; and MIDER, G. B. Potential Sources of Tumor Nitrogen. Cancer Research, **10**: 374–78, 1950.
19. SNEDECOR, G. W. Statistical Methods, p. 75. 4th ed. Ames: Iowa State College Press, 1946.
20. SPERRY, W. M., and WEBB, M. A Revision of the Schoenheimer-Sperry Method for Cholesterol Determination. J. Biol. Chem., **187**:97–106, 1950.
21. STEWART, A. G., and BEGG, R. W. Systemic Effects of Tumors in Force-Fed Rats. II. Effect on the Weight of Carcass, Adrenals, Thymus, Liver, and Spleen. Cancer Research, **13**:556–59, 1953.

Detection of Metastatic Tumor Cells by Intraperitoneal Inoculation of Organ Brei from Tumor-bearing Mice*

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As early as 1889 the pioneer of animal tumor transplantation, A. Hanau (18), recorded the detection of metastases in lymph nodes of a rat by subcutaneous transfer into other rats. Gierke (14) in 1908 attempted "not always successfully" to transfer tumor metastases from the lung of the mouse. The interest in this method increased in 1927 after Blumenthal and Auler (1) interpreted their induction of a subcutaneous tumor by spleen brei from a tumor-bearing rat as indicating the presence of "cancer virus" in apparently normal tissue. Experiments in this direction were continued by several authors (9, 12, 13, 17, 26, 29, 34). Finally, Woglom (40) showed by a crucial demonstration that the presence of even a few metastatic tumor cells in apparently normal tissues accounts for growth of tumors by their inoculation.

It occurred to us that by using the intraperitoneal route for inoculation, it might be possible to avoid the nonspecific tissue reactions often observed in skin injected with organ brei. We have reported previously (15) that intraperitoneally injected mashed organs disappeared from the peritoneal fluid within a few days, while tumor cells of mashed tumors multiplied in this fluid as free cells. Accordingly, we have used the peritoneal cavity as a location that selectively promotes the growth of tumor cells inoculated together with a high number of normal cells. This would seem to make it a favorable site for detection of tumor cells in the blood and organs of mice bearing tumors at various sites of their bodies.

MATERIAL AND METHODS

Tumors and mouse strains.—Two sarcoma strains—S-37 and S-180—were carried in CFW mice in serial intraperitoneal transfers as free cells growing in the peritoneal fluid (15). Carcinoma E 0771 was transferred serially in C57Bl/6 strain of mice by the same method; and mouse carcinoma strains Barrett (C3H/Am) and H-2712 were transferred in C3H mice by using mashed peritoneal implants (owing to a scarcity of tumor cells in the fluid).[1]

Pattern of the experiment.—Groups of mice inoculated with tumor cells of the same strain, by the same route, and designated as "donors of organs" were sacrificed serially, at various time intervals, and bled. Their livers, kidneys, spleens, lungs, and brains were mashed separately, and the brei of each organ, as well as the withdrawn blood, were injected intraperitoneally into new mice. These mice recipients of organ brei were periodically examined for gross evidence of tumor growth in their peritoneal cavity, for the presence of tumor cells in their peritoneal fluid, and, finally, at the autopsy, for tissue characteristics of any implant. Positive results of these examinations—i.e., evidence of tumor growth in the peritoneal cavity—were interpreted as a demonstration of the presence in inoculated material of viable tumor cells spread from the primary tumor growth by implantation and by metastasis.

Inoculation of tumor cells into "donors."—Large numbers of tumor cells (50 to 100 million) were inoculated into prospective "donors of organs" to obtain abundant primary growth. Technics of intraperitoneal and intrapleural inoculation of requisite numbers of cells were described elsewhere (15, 16). For the site of subcutaneous inoculation, we used two areas, topographically distant: (*a*) the flank (left) and (*b*) the scalp. The site of intramuscular injection was the left thigh.

Inoculation of mashed organs from "donors" into "recipients."—Blood of each sacrificed donor was withdrawn with a capillary pipette (about 0.2 ml.) from the right heart and injected intraperitoneally, while still fluid, into the recipient. Organs from serous cavities were rinsed twice in 0.85 per cent NaCl solution before mashing in order to avoid any contamination of the organ brei with serous fluid which might contain tumor cells. In several instances samples of the third rinsing fluid were injected intraperitoneally into new mice, and specimens of peritoneal fluid from these mice were examined at various intervals. In no instance were tumor cells found in these smears. It was presumed, therefore, that any growth from twice-rinsed organs could hardly be attributed to contamination with tumor cells from serous exudate. Each organ was mashed in about 3 ml. of 0.85 per cent NaCl solution, and about 1 ml. of the brei suspension was injected intraperitoneally into the mouse.

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[1] Tumor Strains E 0771 and H-2712 and Mouse Strains C3H and C57Bl/6 were obtained from R. Jackson Memorial Laboratory, Bar Harbor, Maine; CFW Strain from Carworth Farms, New City, New York; Barrett Carcinoma (C3H/Am) through the courtesy of Dr. M. K. Barrett, National Cancer Institute.

Recording of the results.—Recipients of organ brei were usually examined after 10 days, after 15 to 20 days if they survived, and, in rare instances, later. The examination included withdrawal of a specimen of peritoneal fluid for microscopic test, palpation of the abdomen, and, ultimately, the autopsy. We described previously (15) the "auto-inoculation" of the abdominal wall with tumor cells from peritoneal fluid—i.e., tumor tissue growth at the sites of inoculating or exploratory abdominal punctures. The sharply localized ingrowth of tumor cells from the fluid into the serosa and subcutaneous tissue made easier the detection of positive results of organ transfers, in particular, if only a small number of tumor cells were inoculated with the organs. Occurrence of either free tumor cells in the peritoneal fluid or of implants in the peritoneal cavity was recorded as a positive result.

RESULTS

In control experiments none of the CFW, C57, or C3H mice injected intraperitoneally with mashed organs of normal mice presented, at the autopsy (after 7 days), any evidence of new growth or of a tissue reaction. However, their abdomens were often distended by about 0.5 ml. of peritoneal fluid containing mostly macrophages; while in new mice the amount of fluid was not above 0.2 ml. and the proportion of macrophages not more than 15 per cent. These results do not support the contention of some authors (4, 22) that the gross picture of ascites and the resulting increase in body weight may be accepted as criteria for the demonstration of tumor cell growth in peritoneal fluid.

In control CFW mice inoculated with free tumor cells from the peritoneal fluid, i.e., a suspension of tumor cells without any trace of tissue, the peritoneal cavity, after 4–6 days, generally contained a copious amount (about 1 ml.) of fluid containing tumor cells, and eventually a small tumor appeared at the site of puncture. However, in mice inoculated with a mixture of free cells and mashed organs there were, in addition to the above types of growth, several small circular tumors on visceral serosa (20).

In summarizing our experiments, we have recorded for each group of mice bearing primary tumors of the same strain, same age, and same localization, the frequency of intraperitoneal growth from their organs inoculated into new mice; i.e., the frequency in these organs of tumor cells transported from the site of primary growth by implantation and by metastasis. The data on all groups were tabulated separately for each tumor strain. It appears from Tables 1–5 that each tumor strain showed, under similar experimental conditions, a difference in the rate of growth and the frequency and localization of tumor cell spread into organs. Thus, in all groups of mice with primary growth of Sarcoma 37, these cells were detected at an earlier stage after inoculation, in a higher percentage of animals, and in a greater variety of organs than in mice with other tumors (Table 1 as compared to Tables 2–5). The difference between the two sarcoma strains, 37 and 180 was significant, but only quantitative (Tables 1 and 2). However, carcinoma strains (Tables 3, 4, and 5) showed, besides considerable delay in appearance of metastases, some peculiarities of their distribution, such as extreme scarcity of brain metastases from the primary carcinoma E 0771 (Table 3), lack of metastases from the scalp for the primary Barrett carcinoma (Table 4), absence of metastases in abdominal organs from intrapleural carcinoma H-2712 (Table 5), etc.

On the other hand, within this wide range of variations, the following common factors appeared to be significant for metastatic growth from primary tumors of all strains:

a) Site of primary tumor growth.—Organs from donors bearing their primary growth in serous cavities, particularly in the peritoneal cavity, induced intraperitoneal tumors in the majority of recipients. The next highest percentage of tumor growth from organs was recorded for donors with the primary neoplasm in subcutaneous tissues of the flank; a lower frequency was found in bearers of intramuscular tumors, and the poorest results in donors having primary subcutaneous tumors in their scalps, in spite of the large size of these tumors.

b) Age of primary tumor growth.—As a rule, the percentage of positive results of organ inoculation increased with the age of primary tumor growth in donors. For instance, metastatic cells were detected only in later stages of primary carcinoma Barrett or carcinoma H-2712 growth (Tables 4 and 5).

c) Number of tumor cells in organs.—Organs of mice bearing large S-37 tumors in their scalps induced a considerable number of tumors in recipients, but only following a long interval (20 days) after inoculation (Table 1). Since S-37 grows fast, this delay in the growth can be attributed only to the scarcity of viable tumor cells in inoculated organs. Late positive results of organ inoculation were recorded also in some groups with other tumors, in particular for Ca H-2712 (Table 5). Thus, the scarcity of tumor cells in organs was reflected by the delay of their growth into sizable tumors but, apparently, without decrease in their frequency. However, it was noted that slowly growing tumors in recipients had a tendency to become necrotic and to regress. Thus, some instances of initial tumor growth from organs could have been missed.

DISCUSSION

The purpose of this paper was to describe a practical method for serial detection in tumor-bearing mice of metastases in various organs with a minimum of pseudopositive or pseudonegative results; to study with the aid of this method the spread of metastases from primary tumors grown at various sites of the body; and to outline, on the basis of these results, the pattern of localization and frequency of metastases for five different tumor strains.

The principle of detection of metastatic tumor cells by inoculation of organ brei is not new, as it was pointed out in our introduction, but only subcutaneous and intramuscular routes of inoculation were explored and for the most part in a very small series of animals (1, 2, 12, 13, 14, 17, 18, 26, 27, 29). The use of the intraperitoneal route in our

TABLE 1

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF S-37-BEARING CFW MICE

Tumor growth in mice donors of organs			Frequency of tumor growth in mice (Recipients of i.p. injected blood and mash from the following organs of mice donors)					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of mice recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	1	10	0	1	2	0	0	0
	2	11	0	5	6	4	0	0
	3	11	2	8	8	6	4	2
	3	11	4	10	7	8	6	5
	5	11	3	10	8	10	7	4
Pleural cavity	1	10	0	0	0	0	4	0
	2	10	0	0	0	1	4	0
	3	10	4	10	3	9	10	3
	5	11	2	9	4	8	8	2
Subcutaneous tissue:	2	10	0	0	0	0	0	0
	5	11	2	0	3	2	4	0
Flank	10	11	5	6	8	8	4	0
Scalp	11	10	0	0	0	0	0	0
	11	20	3	6	3	3	6	6
Muscle of the thigh	5	10	0	0	0	0	0	0
	15	10	2	0	3	2	4	0
	15	15	5	0	6	8	5	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

TABLE 2

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF S-180-BEARING CFW MICE

Tumor growth in mice donors of organs			Frequency of tumor growth in mice (Recipients of i.p. injected blood and mash from the following organs of mice donors					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of mice recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	1	10	0	0	0	0	0	0
	2	10	0	0	0	0	2	0
	4	10	2	8	6	8	9	4
	6	10	2	4	3	3	7	2
	6	15	0	6	4	3	9	1
Pleural cavity	4	10	0	1	0	0	1	0
	8	10	5	6	3	9	10	2
Subcutaneous tissue:	6	10	0	1	0	1	3	0
Flank	10	10	0	4	6	6	4	0
	12	10	0	6	4	3	6	0
Scalp	8	10	0	0	0	0	2	2
	11	20	0	1	0	0	4	3
Muscle of the thigh	4	10	0	0	0	0	4	0
	4	17	2	0	0	0	6	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

TABLE 3

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF CARCINOMA E 0771-BEARING C57BL/6 MICE

TUMOR GROWTH IN MICE DONORS OF ORGANS			FREQUENCY OF TUMOR GROWTH IN MICE (Recipients of i.p. injected blood and mash from the following organs of mice donors)					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	2	15	0	0	0	0	1	0
	4	15	0	0	0	0	1	0
	10	14	0	4	8	3	7	0
	10	20	0	4	9	3	8	0
Pleural cavity	7	10	0	2	7	6	8	0
Subcutaneous tissue:	3	20	0	0	0	0	0	0
Flank	5	20	0	0	0	0	0	0
	12	10	0	0	0	0	6	0
	12	20	0	6	3	0	7	0
Scalp	13	10	0	0	0	0	4	2
Muscle of the thigh	10	10	0	0	0	0	4	0
	10	20	0	0	0	0	9	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

TABLE 4

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF BARRETT CARCINOMA-BEARING C3H MICE

TUMOR GROWTH IN MICE DONORS OF ORGANS			FREQUENCY OF TUMOR GROWTH IN MICE (Recipients of i.p. injected blood and mash from the following organs of mice donors)					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	12	12	0	8	6	7	7	3
	15	10	0	5	7	8	7	1
	20	10	0	6	4	6	8	0
	30	10	0	8	7	6	8	0
	30	10	0	8	9	9	10	0
Pleural cavity	8	10	0	2	0	0	10	0
	12	10	0	3	1	8	10	0
	12	20	0	7	1	8	10	0
Subcutaneous tissue:	5	10	0	0	0	0	3	0
Flank	12	10	0	0	2	0	8	0
Scalp	15	15	0	0	0	0	0	0
Muscle of the thigh	20	10	0	2	0	0	7	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

TABLE 5

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF CARCINOMA H-2712-BEARING C3H MICE

TUMOR GROWTH IN MICE DONORS OF ORGANS			FREQUENCY OF TUMOR GROWTH IN MICE (Recipients of i.p. injected blood and mash from the following organs of mice donors)					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	20	10	0	3	4	2	8	0
	20	20	0	8	5	7	9	0
Pleural cavity	20	20	0	0	0	0	5	0
Subcutaneous tissue:	20	10	0	0	0	0	0	0
Flank	20	20	0	1	0	2	6	0
Scalp	20	20	0	2	0	0	5	0
Muscle of the thigh	20	20	0	1	0	0	3	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

experiments eliminated nonspecific reactions in subcutaneous or muscle tissue (swelling, necrosis, granulations, scar formation) which frequently obliterate growth from small inocula or simulate a tumor in negative cases. Moreover, the growth of tumor cells from intraperitoneally inoculated material could be identified, in the majority of mice, by rapid examination of smears from peritoneal exudate. Thus, it was technically easy to obtain clear-cut results in a large number of mice serially inoculated with brei of various organs. Furthermore, primary tumors were grown in mice donors of organs at various body sites selected to produce lymph-borne metastases (by inoculation of the flank), blood-borne metastases (from scalp tumor), or serous fluid-borne implantation and metastases (from free tumor cell growth in the peritoneal or the pleural exudate).

It should be pointed out that our positive results constitute some evidence that a sufficient number of cells from primary neoplasms have reached several organs (7), but they do not indicate whether these tumor cells started to proliferate before their transfer with the organ brei into new mice. Moreover, our negative results do not exclude the occurrence in the organ of a very low number of tumor cells or their inhibition by a defense reaction in the recipient (3, 19, 37).

Our data illustrate the spread of metastatic cells from various initial points (various sites of primary neoplasms of various strains) to various end points (various organs) and suggest the analogy between these results and the data of human pathology.

a) Fluid-borne implantation and metastases.—It is understandable that intraperitoneally inoculated tumor cells floating in the peritoneal fluid penetrated into open stomata between the cavity and the lymphatics (10) or nestled below the visceral peritoneum by damaging the lining, inducing a coagulum and stimulating an ingrowth of fibroblasts (30). It is possible that tumor cell implantation from serous fluid on the surface of abdominal organs resulted eventually in metastases comparable to transcoelomic metastases of human tumors (39), while in other cases there was only local invasion of the organ surface. This problem is under investigation.

b) Lymph-borne metastases.—Serial autopsies of mice with flank tumors showed progressive infiltration of lymphatics and lymph nodes in the abdominal wall with tumor growth reaching peritoneal serosa in most animals. This process seemed to be analogous to the spread of human metastases by "lymphatic permeation" or by embolization (39).

c) Blood-borne metastases.—Tumors were grown from the blood of mice bearing S-37 cells at any site of their bodies, mostly in later stages of their growth (Table 1), less often in mice with S-180 growth, and never in animals inoculated with carcinoma strains. Previous reports on occurrence of S-37 cells in the blood (21, 29) and of cells from other sarcoma strains (24, 25) showed extensive variations in the frequency of these results, depending apparently on the age and the site of primary tumors (Table 1). Our negative results with carcinoma strains did not exclude an intermittent occurrence, at long time intervals, of tumor emboli in the blood. Indeed, metastases in the lungs and in the liver from carcinoma growing in the scalp were obviously blood-borne, and so, probably, were the metastases from the thigh. The observation that they were localized frequently in the lung, rarely in the liver, and not at all in other organs (Tables 3, 4, and 5) is in agreement with the known tendency of human tumor emboli liberated into the blood stream to be arrested by the first capillary bed of the systemic blood in the lung and those of portal blood by the liver (28, 35, 39).

Our results agree with observations of early workers on the localization of metastases from slowly growing subcutaneous mouse tumors mainly in the lungs, and exceptionally in the liver (14, 27), on the distribution of metastases in animals after intravenous injection of tumor cells (23, 31–33, 36), and on high frequency of implantation and metastases from intraperitoneally inoculated sarcoma (25). While our results support the hypothesis that local tissue invasion is a prerequisite to the formation of blood-borne metastases (8) and that localization of metastases depends primarily on the availability of tumor emboli and the anatomical distribution of the vascular system (6, 7, 8, 11, 28), our data may suggest that the availability of tumor emboli depends on the opportunities for tumor cells or clumps to get separated, owing to their reduced adhesiveness (5), from the neoplastic tissue, as in serous fluid or in edematous connective tissue. Moreover, our results may be interpreted as illustrating a parallelism between the growth rate of neoplasms and their tendency to induce metastases.

The method outlined above for detection of metastatic cells may be used for the study of the mechanism of metastatic processes in transferable mouse tumors. Moreover, it may be applied for screening the effect of various physical and chemical agents on tumor cells transported into organs from the primary growth. We are studying the effect of radioactive colloidal gold on metastatic

tumor cells in mice, and the results will be reported separately.

SUMMARY AND CONCLUSIONS

1. Intraperitoneal inoculation of mashed organs or blood from tumor-bearing mice resulted, in numerous instances, in peritoneal growth in new mice. These results were attributed to the presence in the inoculated material of viable tumor cells spread from the primary growth by implantation and by metastasis.

2. By the use of this method in mice bearing tumors of various strains (two sarcomas: S-37 and S-180; three carcinomas: E 0771, Barrett's [C3H/Am] and H-2712) at various sites of their bodies, it was found that the frequency and distribution of metastatic cells in various organs showed different patterns for various tumor strains and depended, for the same tumor strain, on the site and the age of primary tumor growth. The number of metastatic cells in an organ was reflected by the rate of growth of intraperitoneal tumors induced by inoculation of this organ.

3. Discussion of the data correlating the site of localization of metastatic cells (certain organs) with the site of their origin (site of the primary neoplasm) indicated the spread of these cells from the primary tumor by routes of serous fluids, lymph, and blood.

4. Application of the outlined method of detection of metastatic cells for the study of metastases from transferable tumors and for the screening of metastases-inhibiting agents is suggested.

REFERENCES

1. BLUMENTHAL, F., and AULER, H. Mit Milzbrei von Tumorratten erzeugter Tumor. Ztschr. f. Krebsforsch., **24**:285–91, 1927.
2. BONNE, C. Fréquence des métastases pulmonaires dans les cas de tumeurs greffes dans la queue de la souris. Compt. rend. Soc. de Biol., **93**:312–14, 1925.
3. BROWN, W. H., and PIERCE, L. Studies Based on a Malignant Tumor of the Rabbit. V. Metastases. J. Exper. Med., **38**:349–405, 1923.
4. COLLIER, W. A. Ueber den Nachweis des Agens des Ehrlichschen Ascitescarcinoms in den Organen der Maus. Ztschr. f. Krebsforsch., **41**:303–6, 1934.
5. COMAN, D. R. Decreased Mutual Adhesiveness, a Property of Cells from Squamous Cell Carcinoma. Cancer Research, **4**:625–29, 1944.
6. ———. Mechanisms Responsible for the Origin and Distribution of Blood-borne Tumor Metastases. *A Review. Ibid.*, **13**:397–404, 1953.
7. COMAN, D. R.; DE LONG, R. P.; and MCCUTCHEON, M. Studies on the Mechanism of Metastasis. The Distribution of Tumors in Various Organs in Relation to the Distribution of Arterial Emboli. Cancer Research, **11**:643–51, 1951.
8. COMAN, D. R.; EISENBERG, R. B.; and MCCUTCHEON, M. Factors Affecting the Distribution of Tumor Metastases, Experiments of V_2 Carcinoma of Rabbits. Cancer Research, **9**:649–51, 1949.
9. COSTA, A. Versuche über die Übertragung der experimentellen Tumoren der Huehner und Säugetiere durch Gehirnbrei von an Tumor erkrankten Tieren. Ztschr. f. Krebsforsch., **36**:233–44, 1932.
10. CUNNINGHAM, R. S. The Physiology of the Serous Membranes. Physiol. Rev., **6**:241–81, 1926.
11. DE LONG, R. P., and COMAN, D. R. Studies of Metastases. The Effect of Anatomical Location on the Growth of Tumor Implants. Cancer Research, **10**:212, 1950.
12. FLAKS, J. Untersuchungen über Metastasen. II. Teil über Transplantation einiger Organe der Tumorratten. Ztschr. f. Krebsforsch., **37**:504–9, 1932.
13. FRAENKEL, E. Beitraege zur Organtherapie von Neoplasmosen. Ztschr. f. Krebsforsch., **37**:313–22, **38**:394–97, 1932.
14. GIERKE, E. The Hemorrhagic Mammary Tumors. Sc. Rep. Invest. Imp. Cancer Res. Fund, **3**:115–45, 1908.
15. GOLDIE, H., and FELIX, M. Growth Characteristics of Free Tumor Cells Transferred Serially in the Peritoneal Fluid of the Mouse. Cancer Research, **11**:73–80, 1951.
16. GOLDIE, H.; JEFFRIES, B. R.; MAXWELL, M. C.; and HAHN, P. F. Growth of Free Tumor Cells in the Pleural Exudate and Their Implantation into the Pleura of the Mouse. Cancer Research, **12**:422–25, 1952.
17. GROSS, L. Über die Zeitgrenzen der Übertragungsfähigkeit eines Impfsarkoms mittels des Blutes und der tumorfreien Organe der Maus. Ztschr. f. Krebsforsch,. **38**:241–48,1932.
18. HANAU, A. Erfolgreiche experimentelle Übertragung von Karzinom. Fortschr. d. Med., **9**:5–12, 1889.
19. IWASAKI, T. Histological and Experimental Observations on the Destruction of Tumor Cells in the Blood Vessels. J. Path. & Bact., **20**:85–105, 1915–16.
20. JONES, F. S., and ROUS, P. On the Cause of Localization of Secondary Tumors at Points of Injury. J. Exper. Med., **20**:404–12, 1914.
21. JONESCU, P. Über das Vorkommen von Geschwulstellen in strömendem Blut von Tieren mit Impftumoren. Ztschr. f. Krebsforsch., **33**:264–80, 1930.
22. LETTRÉ, H. Einige Versuche mit dem Mäuseascites Tumor. Ztschr. f. Krebsforsch., **57**:1–13, 1950.
23. LEVIN, I., and SITTENFIELD, M. J. On the Mechanism of the Formation of Metastases in Malignant Tumors, an Experimental Study. J. Exper. Med., **14**:148–58, 1911.
24. LIPSCHITZ, B. Erzeugung von Tumoren mit Blut von Tumortieren. Wiener Klin. Wchnschr., **42**:1593–94, 1929.
25. MAZZACUVA, G. Condizioni determinanti la producione di metastasi da innesti di sarcoma galliera. Pathologica, **25**: 856–68, 1933. "Abstr.," Am. J. Cancer, **25**:825, 1935.
26. MONTANIS, Y. S. Reproduction du lymphosarcome transplantable de la souris par inoculation du sang et d'organes des animaux porteurs de la tumeur. Compt. rend. Soc. de Biol., **106**:371–72, 1931.
27. MURRAY, J. A. Spontaneous Cancer in the Mouse. Histology, Metastasis, Transplantability, and the Relations of Malignant New Growths to Spontaneously Affected Animals. Sc. Rep. Invest. Imp. Cancer Res. Fund, **3**:69–114, 1908.
28. NETTLESHIP, A. Basic Principles of Cancer Practice, pp. 86–87. Baltimore: Williams & Wilkins Co., 1952.
29. PARSONS, L. D. Changes in the Lymph Glands of Tumor-bearing Mice. J. Path. & Bact., **47**:501–23, 1938.
30. SAMPSON, A. A. The Origin and Significance of Newly Formed Lymph Vessels in Carcinomatous Peritoneal Implants. Am. J. Pathology, **12**:437–67, 1936.
31. SCHMIDT, W. Über die Bildung von Lymphknotene-

metastasen beim Ascitescarcinom der Maus. Ztschr. f. Krebsforsch., **48**:506–8, 1938.

32. Sugarbaker, E. D. The Organ Selectivity of Experimentally Induced Metastases in Rats. Cancer, **5**:606–12, 1952.
33. Takahashi, M. An Experimental Study of Metastasis. J. Path. & Bact., **20**:1–13, 1915–16.
34. Tinozzi, F. P. Weitere Versuche zur Erseugung von Tumoren mit Embryonalbrei von Tumortieren. Ztschr. f. Krebsforsch., **36**:193–204, 1932.
35. Walther, E. Krebsmetastasen. Basel: B. Schwalbe, 1948.
36. Warren, S., and Gates, O. The Fate of Intravenously Injected Tumor Cells. Am. J. Cancer, **27**:485–92, 1936.
37. Weil, R. The Intravascular Implantation of Rat Tumor. J. Med. Research, **28**:497–508, 1917.
38. Wibeau, F., Über Metastasenbildung nach intravenouser Injection carcinomatöser Ascitesfluessigkeit von Mäusen und Ratten. Ztschr. f. Krebsforsch., **39**:66–71, 1933.
39. Willis, R. A. The Spread of Tumors in the Human Body. London: J. A. Churchill, 1934.
40. Woglom, W. H. Metastasis to the Lymph Nodes from Mouse Sarcoma 37. Am. J. Cancer, **38**:328–34, 1940.
41. Zeidman, I.; McCutcheon, M.; and Coman, D. R. Factors Affecting the Number of Tumor Metastases, Cancer Research, **10**:357–59, 1950.

Occurrence of Pulmonary Tumors in Strain A Mice Following Total-Body X-Radiation and Injection of Nitrogen Mustard

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One of the sources of evidence for the somatic mutation hypothesis of cancer is in studies of correlation between mutagenic and carcinogenic capacities of physical and chemical agents. Tests in this laboratory have suggested a positive correlation within the limits of three mustard compounds. Both the nitrogen mustard, methylbis (2-chloroethyl)amine hydrochloride, and sulfur mustard, bis(2-chloroethyl) sulfide, were shown to be strong carcinogens in the induction of pulmonary tumors when injected intravenously into strain A mice (9, 10). Both had been demonstrated to be strong mutagens when tested on Drosophila and microorganisms,[1] and recently Falconer, Slizynski, and Auerbach (6) have reported genetic changes in the progeny of mice treated with nitrogen mustard. On the other hand, mustard oil, allyl iso-thiocyanate, that Auerbach and Robson (3) found to be a weak mutagen when tested on Drosophila, did not significantly increase the occurrence of pulmonary tumors in strain A mice.

Such studies are logically extended to the observation of the effect of combining agents which individually have carcinogenic effects and for which additive mutagenic effects have been demonstrated. Radiation and the mustards comprise such combinations. The carcinogenic action of radiation has long been established, and, in the work of Lorenz *et al.* (21), an increase in the occurrence of lung tumors in strain A mice was observed following exposure to chronic gamma radiation, and later such an increase was also observed following fractionated acute x-radiation (18). Aside from the demonstration of an increase in pulmonary tumors in strain A mice injected with nitrogen and sulfur mustard (9, 10), carcinogenic action of nitrogen mustards has also been demonstrated by Boyland and Horning (5) for other strains, and by Griffin, Brandt, and Tatum (7) for rats as well as for mice. Additive mutagenic effects of radiations and mustards have been observed by a number of investigators. Auerbach (1) observed an additive effect of x-radiation and mustard gas in increasing mutation rate in Drosophila. Swanson and Goodgal (25) found that pretreatment with nitrogen mustard increased the mutagenic effect of ultraviolet light on Aspergillus, and Swanson, McElroy, and Miller (26) observed a similar effect in Neurospora. Kaufmann, Gay, and Rothberg (16) recorded an increase in chromosomal rearrangements in Drosophila treated with nitrogen mustard and pretreated with infrared radiation over that in those treated with nitrogen mustard alone. With such observations as a background, a project was outlined and carried out for ascertaining whether or not an additive effect of x-radiation and nitrogen mustard could be demonstrated in the induction of pulmonary tumors in strain A mice.

MATERIALS AND METHODS

The mice used in these studies were the highly inbred strain A that has been used extensively in studies on pulmonary tumors. The incidence of pulmonary tumors in untreated mice of this strain is approximately 90 per cent in animals 18 months of age and 50 per cent in animals 12 months of age (8). No sex difference in incidence has been observed.

Two hundred fifty-six strain A mice 2 and 3 months of age and equally divided as to sex were distributed in four groups; group I, the controls; group II, those to receive x-radiation; group III, those to receive nitrogen mustard; and group IV, those to receive x-radiation plus nitrogen mustard. Littermates were distributed among the four groups. The animals were kept in plastic cages with eight animals in each cage and with the sexes separated. They were fed Derwood pellets and were given an unlimited supply of tap water.

The mice of groups II and IV were given a single total-body exposure of 900 r x-radiation. The irradiation conditions were: 186 kvP, 20 m. amp., 0.25 mm. Cu + 1.06 mm. Al. Two tubes opposite each other were used to obtain a uniform tissue dose; their foci were 54 cm. from the center of the mice.

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[1] For review see C. Auerbach (2).

They gave a dosage rate of 93 r per minute. Since this dose of 900 r is 100 per cent lethal, the spleens of the mice were surgically exteriorized and shielded in leaden chambers according to the procedure described by Jacobson and co-workers (12). This procedure effectively protects against the lethal effects of such a dose.

At the same time that the mice of groups II and IV were irradiated, those of groups I and III were subjected to a mock spleen-shielding operation to make their treatment comparable with the irradiated groups in this respect. Ten days later, when the animals had recovered sufficiently from the spleen operation and irradiation to receive nitrogen mustard, those of group III, that had been subjected to the mock spleen-shielding operation, and those of group IV, that had been irradiated with their spleens shielded, were each given an injection in the caudal vein of 0.025 mg. nitrogen mustard, methylbis(2-chlorethyl)amine hydrochloride, in 0.25 cc. distilled water. Two days later these animals were given a second similar injection.

TABLE 1

OCCURRENCE OF PULMONARY TUMORS IN MICE TREATED WITH X-RADIATION AND NITROGEN MUSTARD

Group	Treatment	Months after treatment*									Total	Per cent with tumors	Av. no. nodules	Av. no. months after treatment
		3	4	5	6	7	8	9	10	11				
I	Control		0/1	0/2	1/5	3/9	0/4	3/5	1/6	17/32	25/64	39	0.7	9.3
II	X-radiation	0/2	1/3	0/3	0/5	0/5	2/9	1/11	2/3	7/16	13/55	24	0.6	8.4
III	HN_2			1/1	1/1	3/4	9/10	10/10	7/8	24/25	55/59	93	4.2	9.6
IV	X-radiation+ HN_2	0/1	1/1		1/7	1/7	0/5	5/11	2/6	11/17	21/55	38	1.1	8.8

* Number with tumors/number autopsied.

All animals were weighed at the time of the spleen operation and irradiation, 3 days later, at the time of each injection, 2 weeks later, and, subsequently, at 4-week intervals.

Approximately 9 per cent of the animals died within a few days after the treatment or died at a later date and were eaten by their cage mates or were too autolyzed for examination. Autopsy records were obtained on all other animals when they were found dead or moribund during the course of the experiment or at 11 months after the spleen operation and irradiation, at which time all remaining animals were killed and examined. Any lung nodules or other neoplasms that were found were saved for histologic examination, as were the sternum, a lymph node, spleen, ovaries or testes of each animal, and the eyes of the irradiated animals. Tissues from about half of the animals were fixed in Fekete's modification of Tellyesniczky's fluid,[2] and those from the other animals were fixed in a fluid[3] devised for decalcifying the bone. All sections were stained with hematoxylin and eosin.

RESULTS

The occurrence of pulmonary tumors in the animals of the four groups is listed in Table 1. In the 64 animals of the control group I, pulmonary tumors were found in twelve of the 32 males and in thirteen of the 32 females, or in 39 per cent of the total number of animals. The over-all mean number of nodules for the group was 0.7. In group II, which received x-radiation alone, pulmonary tumors were observed in seven of the 27 males and in six of the 28 females, i.e., 24 per cent of the total number. The mean number of nodules in this group was 0.6. Thus, the irradiated animals did not show an increase in occurrence of pulmonary tumors, as could have been expected from previous observations of irradiated strain A mice. The incidence was actually lower in the irradiated group, but the difference was not statistically significant; $X^2 = 3.24$; P is between 0.05 and 0.10. Nor was the difference between the mean number of nodules significant; $t = 0.645$; P is between 0.5 and 0.6.

In group III, which received nitrogen mustard alone, pulmonary tumors occurred in 27 of the 29 females and in 28 of the 30 males—or in 93 per cent of the total number. This incidence was significantly greater than that in the control group; $X^2 = 39.6$; $P < 0.01$; and significantly greater than that in the group receiving x-radiation alone; $X^2 = 59.8$; $P < 0.01$. The average number of tumors in the group receiving nitrogen mustard alone was 4.2, which was significantly greater than that in the control group; $t = 7.09$; $P < 0.01$; and significantly greater than that in the group receiving x-radiation alone; $t = 7.11$; $P < 0.01$.

Group IV, which received x-radiation plus nitrogen mustard, showed not only no increase in occurrence of pulmonary tumors over that in the group receiving nitrogen mustard alone, but also a decrease to approximately the incidence in the control group. Eight of the 24 females and thirteen of the 31 males, or only 38 per cent of the total number in group IV had pulmonary tumors. This incidence was significantly less than that in group III; $X^2 = 38.8$; $P < 0.01$; but not significantly different from that in the control group I; $X^2 = 0.0097$; P is between 0.90 and 0.95; or from that in group II; $X^2 = 2.72$; P is between 0.05 and 0.1. The average number of nodules in the irradiation

[2] 70 per cent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part.

[3] This fixative formulated by W. D. Levillain consisted of 70 per cent ethyl alcohol, 200 cc.; formalin, 20 cc.; trichloroacetic acid, 10 gm.; urea, 0.8 gm.

plus nitrogen mustard group was 1.1 which was significantly less than that of group III; $t = 6.138$; $P < 0.01$; but not significantly greater than that of the control group; $t = 1.37$; P is between 0.1 and 0.2; or that of group II; $t = 1.73$; P is between 0.05 and 0.1.

Neoplasms of the blood-forming organs occurred in eight animals of groups I, II, and III.[4] One male and one female of the x-radiated group had reticulum-cell sarcomas at 7 and 10 months, respectively, after treatment. Another female of the same group at 6 months postirradiation had what was classified as lymphocytic leukemia; it was atypical in that the cells were large with indented or bean-shaped nuclei and with relatively more cytoplasm than is characteristic of typical lymhocytic leukemia. Furthermore, the cells were more clumped than in the typical lymphocytic leukemia. A fourth animal, a female, of the x-radiated group had what was probably a Hodgkin's-like lesion at 9 months postirradiation, but because of post mortem changes, this diagnosis was uncertain. Two females of the group receiving nitrogen mustard alone had what was classified as Hodgkin's-like lesions at 5 and 11 months after the beginning of the experiment. One female of the control group developed a lymphocytic leukemia 7 months after the experiment was set up. This, also, was atypical, resembling the Hodgkin's-like lesions. No neoplasms of the blood-forming organs occurred in the animals receiving x-radiation plus nitrogen mustard.

It may be significant that, of all these neoplasms of the blood-forming organs, none was classified as a typical lymphocytic leukemia. It should also be noted that neither x-radiation nor nitrogen mustard significantly increased the incidence of this type of neoplasm. Examination of the hematopoietic tissues of the animals without such neoplasms failed to show any significant difference between the control group and either of the three treated groups. These observations are in line with those of Jacobson *et al.* (13), who found complete recovery in the hematopoietic tissue of mice whose spleens were protected during irradiation with a lethal dose.

The only other neoplasm occurring in any animals of this experiment was a small sarcoma, probably a fibrosarcoma, involving the xiphoid process of a control female, at 11 months after the beginning of the experiment.

A rough estimate was made of the atrophy of the spermatogenetic elements of the tubules of the testes. Some atrophy was noted in the controls. The changes were slightly more advanced in those receiving nitrogen mustard alone, considerably more advanced in those receiving x-radiation alone, and most advanced in those receiving x-radiation + nitrogen mustard. The reduction of a number of viable sperm in the epididymides of the four groups paralleled the destruction of the elements in the tubules.

A sharp distinction could be made between the ovaries of animals that had received radiation and of those that had not. None of the ovaries of the females receiving x-radiation or x-radiation plus nitrogen mustard showed any growing follicles, whereas growing follicles were observed in the ovaries of all of the control group and of the group receiving nitrogen mustard alone, except for one female of the latter group. No ovarian tumors were observed, but the experiment was terminated at 11 months following irradiation, whereas the latent period for the irradiation-induced ovarian tumors is longer (22).

The animals of group II receiving irradiation alone and those of group IV receiving irradiation plus nitrogen mustard all developed ocular lesions. These were comparable to those occurring in strain A mice that received 400 r x-radiation to the whole body at birth and described by Lorenz and Dunn (20).

Weight curves of the four groups are shown in Chart 1. There was a reduction in weight following the spleen-shielding operation and irradiation and also following the mock spleen-shielding operation and again following the injection of nitrogen mustard. The weight losses were regained in all four groups, with the control group and the group receiving nitrogen mustard alone gaining considerably more weight than the groups receiving x-radiation and x-radiation plus nitrogen mustard.

DISCUSSION

This experiment did not reveal an additive effect of radiation and nitrogen mustard in the induction of pulmonary tumors in mice comparable to the additive effect of the two agents in the induction of mutations in Drosophila and other lower organisms. However, the 900 r x-radiation, as it was applied in this experiment with the spleens of the mice shielded, did not itself increase the occurrence of pulmonary tumors. Had the nitrogen mustard been given along with radiation administered chronically or in divided doses as had been formerly shown to be carcinogenic, an additive effect might have been revealed. The data do, however, reveal interesting facts and raise a number of interesting questions. Some of these

[4] The authors gratefully acknowledge the assistance of Dr. Thelma B. Dunn, who examined these sections and concurred in the diagnoses.

must be clarified before support for or contradiction of the somatic mutation hypothesis for the origin of cancer can be claimed.

It is important to note the failure in this study to obtain an increase in occurrence of pulmonary tumors with radiation alone. In a previous study strain A mice exposed to 8.8 r per 8 hours each day until they had received a total dose of 2,400 r had a significant increase in incidence of pulmonary tumors over that of the controls (21). In the present study, however, a single dose of 900 r was given, and the spleens were shielded during the irradiation. This might suggest that the shielding of the spleen may have some inhibiting effect on the carcinogenic action of the irradiation. This idea is supported by recent findings of Lorenz *et al.* (19). Against this assumption, however, is the finding of Lorenz (17) that a significant increase in lung tumors is also found if the total body or the lungs only are irradiated daily with 75 r to a total dose of 750 r. This indicates a direct effect of the irradiation. Moreover, in the experiments in which the lungs only were irradiated, the spleens of the animals were shielded. It seems more likely, therefore, that the massive dose of 900 r may have caused changes in the lungs inhibiting tumor induction. Further studies must be done in comparing the occurrence of pulmonary tumors in animals irradiated, as in the present experiment, with that in those receiving the same total dose in divided exposures without spleen protection.

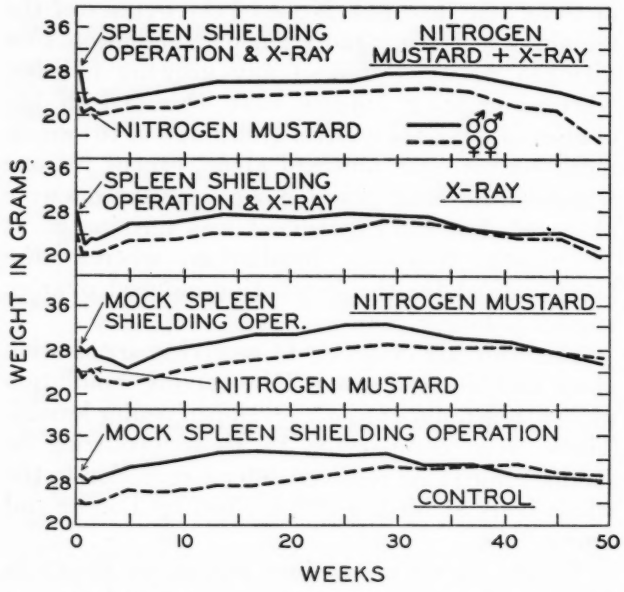

CHART 1.—Weight curves of the experimental and control mice.

It will also be well to determine whether or not clamping off the splenic pedicle during injection of nitrogen mustard has any effect upon the induction of tumors with this compound, since Jacobson *et al.* (14) have shown that this procedure protects against the lethal action of nitrogen mustard. No effect of the splenic operation was apparent in the present experiment, but the operation was done 10 days prior to the injection of the nitrogen mustard. In earlier studies (9, 10), twice the amount of nitrogen mustard that was injected in the present study resulted in approximately twice the pulmonary tumor response, i.e., an average of 3.5 nodules per animal 3–4 months after the injection and seven to nine nodules per animal 10 months after the injection.

The failure to increase the occurrence of pulmonary tumors with radiation in the present experiment, whereas an increase was observed in former studies, might also have been owing in part to the fact that in the present experiment the irradiated animals showed a reduction in body weight as compared to the controls and to those receiving nitrogen mustard alone, whereas the chronically irradiated animals of the earlier experiment did not show a reduction in body weight. A number of factors that reduce body weight have been shown to reduce the occurrence of pulmonary tumors in mice (11). It is doubtful, however, that the difference in weight could have caused the total inhibition observed.

Explanation of the apparent inhibitory effect of the previous irradiation upon the carcinogenic action of the nitrogen mustard is not apparent. The results, however, might have been varied could there have been a different sequence and time interval of the treatments, but these were dictated by the effects of the treatments upon the animals. The animals were unable to withstand the spleen-shielding operation and irradiation when previously injected with nitrogen mustard, and after spleen-shielding and irradiation it was necessary to wait 10 days before the animals could tolerate the nitrogen mustard. The order of treatment might be reversed or the time interval shortened by reducing considerably the amount of nitrogen mustard injected.

The inhibitory effect observed, however, is in line with certain observations on the action of two chemical carcinogens combined. Richardson *et al.* (23) observed an anticarcinogenic effect of 20-methylcholanthrene against the induction of liver tumors in rats with 3′-methyl-4-dimethylaminoazobenzene when the two carcinogens were administered simultaneously. Jaffé (15) had previously not observed any influence of 20-methylcholanthrene or of ethyl urethan on the carcinogenic action of the azo compound, but he injected a much smaller dose of methylcholanthrene than did Richardson and co-workers. Lacassagne *et al.* (17) observed an inhibition by a number of weakly

active carcinogenic hydrocarbons upon the action of stronger carcinogenic hydrocarbons when the weaker compound was administered with the stronger one. Earlier, Berenblum (4) had shown that the induction of warts in mice by repeated application of tar could be almost completely prevented by the addition of mustard gas to the tar. A preliminary experiment in this laboratory, however, has shown an additive effect of dibenzanthracene and nitrogen mustard in the induction of pulmonary tumors.

Rusch *et al.* (24) have studied the effect of the application of ultraviolet radiation in conjunction with carcinogenic hydrocarbons on the induction of papillomas and carcinomas of the skin in mice. While they did not get an inhibition of the action of the hydrocarbon by the irradiation in a degree comparable to that observed in the present study, they failed to get an additive effect of the radiation and the chemical carcinogen.

SUMMARY

The occurrence of pulmonary tumors in strain A mice that had been exposed to 900 r x-radiation with the spleens shielded was comparable to that in control animals that had been subjected only to a mock spleen-shielding operation.

There was a significant increase in the occurrence of pulmonary tumors in strain A mice that had been injected with 0.05 mg. nitrogen mustard following a mock spleen-shielding operation.

In strain A mice that had received 900 r x-radiation with their spleens shielded and subsequently had been injected with 0.05 mg. nitrogen mustard, the occurrence of pulmonary tumors was not significantly different from that of the controls or that of the group receiving irradiation alone and was significantly less than that of the group receiving nitrogen mustard alone.

REFERENCES

1. AUERBACH, C. Chemically Induced Mosaicism in *Drosophila melanogaster*. Proc. Roy. Soc. Edinb. B, **62**:211–21, 1946.
2. ———. Chemical Mutagenesis. Biol. Rev., **24**:355–91, 1949.
3. AUERBACH, C., and ROBSON, J. M. Production of Mutations by Allyl Isothiocyanate. Nature, **154**:81–82, 1944.
4. BERENBLUM, I. The Modifying Influence of Dichloroethyl Sulphide in the Induction of Tumors in Mice by Tar. J. Path. & Bact., **32**:425–34, 1929.
5. BOYLAND, E., and HORNING, E. S. Induction of Tumors with Nitrogen Mustards. Brit. J. Cancer, **3**:118–23, 1949.
6. FALCONER, D. S.; SLIZYNSKI, B. M.; and AUERBACH, C. Genetical Effects of Nitrogen Mustard in the House Mouse. J. Genetics, **51**:81–88, 1952.
7. GRIFFIN, A. C.; BRANDT, E. L.; and TATUM, E. L. Nitrogen Mustards as Cancer-inducing Agents. J.A.M.A., **144**:571, 1950.
8. HESTON, W. E. Inheritance of Susceptibility to Spontaneous Pulmonary Tumors in Mice. J. Nat. Cancer Inst., **3**:79–82, 1942.
9. ———. Induction of Pulmonary Tumors in Strain A Mice with Methyl-bis (β-Chloroethyl) Amine Hydrochloride. *Ibid.*, **10**:125–30, 1949.
10. ———. Carcinogenic Action of the Mustards. *Ibid.*, **11**: 415–23, 1950.
11. HESTON, W. E.; DERINGER, M. K.; HUGHES, I. R.; and CORNFIELD, J. Interrelation of Specific Genes, Body Weight, and Development of Tumors in Mice. J. Nat. Cancer Inst., **12**:1141–57, 1952.
12. JACOBSON, L. O.; MARKS, E. K.; ROBSON, M. J.; GASTON, E.; and ZIRKLE, R. E. The Effect of Spleen Protection on Mortality Following X-Irradiation. J. Lab. & Clin. Med., **34**:1538–43, 1949.
13. JACOBSON, L. O.; SIMMONS, E. L.; MARKS, E. K.; ROBSON, M. J.; BETHARD, W. F.; and GASTON, E. O. The Role of the Spleen in Radiation Injury and Recovery. J. Lab. & Clin. Med., **35**:746–70, 1950.
14. JACOBSON, L. O.; SIMMONS, E. L.; MARKS, E. K.; ROBSON, M. J.; GASTON, E. O.; and BETHARD, W. F. Progress Report: Spleen-shielding Experiments. Quart. Rep., Div. of Biol. & Med. Research, Argonne National Laboratory, pp. 22–29. A. M. BRUES (ed.). ANL-4451. February, March, April, 1950.
15. JAFFÉ, W. G. The Response of Rats to the Simultaneous Application of Two Different Carcinogenic Agents. Cancer Research, **7**:113–16, 1947.
16. KAUFMANN, B. P.; GAY, H.; and ROTHBERG, H. The Influence of Infrared Radiation on the Production by Nitrogen Mustard of Chromosome Rearrangements in *Drosophila*. J. Exper. Zool., **111**:415–36, 1949.
17. LACASSAGNE, A.; BUU-HOÏ; and RUDALI, G. Inhibition of the Carcinogenic Action Produced by a Weakly Active Carginogenic Hydrocarbon. Brit. J. Exper. Path., **26**: 5–12, 1945.
18. LORENZ, E. Some Biologic Effects of Long Continued Irradiation. Am. J. Roentgen. & Radium Therapy, **63**: 176–85, 1950.
19. LORENZ, E.; CONGDON, C. C.; and UPHOFF, D. Prevention of Irradiation-induced Lymphoid Tumors in C57BL Mice by Spleen Protection. Proc. Am. Assoc. Cancer Research, **1**:34–35, 1953.
20. LORENZ, E., and DUNN, T. B. Ocular Lesions Induced by Acute Exposure of the Whole Body of Newborn Mice to Roentgen Radiation. Arch. Ophthalmology, **43**:742–49, 1950.
21. LORENZ, E.; HESTON, W. E.; DERINGER, M. K.; and ESCHENBRENNER, A. Increase in Incidence of Lung Tumors in Strain A Mice Following Long Continued Irradiation with Gamma Rays. J. Nat. Cancer Inst., **6**:349–53, 1946.
22. LORENZ, E.; HESTON, W. E.; ESCHENBRENNER, A. B.; and DERINGER, M. K. Biological Studies in the Tolerance Range. Radiology, **49**:274–85, 1947.
23. RICHARDSON, H. L.; STIER, A. R.; and BORSOS-NACHTNEBEL, E. Liver Tumor Inhibition and Adrenal Histologic Responses in Rats to Which 3′-Methyl-4-Dimethylaminoazobenzene and 20-Methylcholanthrene Were Simultaneously Administered. Cancer Research, **12**:356–61, 1952.
24. RUSCH, H. P.; KLINE, B. E.; and BAUMANN, C. A. The Nonadditive Effect of Uultraviolet Light and Other Carcinogenic Procedures. Cancer Research, **2**:183–88, 1942.
25. SWANSON, C. P., and GOODGAL, S. H. The Effect of Nitrogen-Mustard on the Ultraviolet-Induced Mutation Rate in *Aspergillus terreus*. Genetics, **33**:127, 1948.
26. SWANSON, C. P.; MCELROY, W. D.; and MILLER, H. The Effect of Nitrogen Mustard Pretreatment on the Ultra-Violet-Induced Morphological and Biochemical Mutation Rate. Proc. Nat. Acad. Sc., **35**:513–18, 1949.

Attempts To Induce Immunity against Mammary Adenocarcinoma in Inbred Mice

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INTRODUCTION

The literature on "tumor immunity" has been reviewed by Spencer (14), Woglum (18), and, more recently, by Hauschka (9). Most of the investigations were made with tumors transplanted either into random-bred rats or into strains of mice alien to the strain in which the neoplasm first occurred. In several instances, however, immunity was produced against tumors which arose in inbred strains of rats (10, 11) and mice (6, 12). In the experiments by Lewis *et al.* (10, 11), immunity to uniformly progressive tumors of inbred rats was produced following autolysis of the tumor as a result of surgical ligation of its blood supply or by its strangulation. These results and those of Gross (6, 7) were explained by the assumption that the tumor had mutated during repeated transplantations (9, 13). It was of interest to repeat the ligation experiments of Lewis *et al.* with strain-specific tumors in inbred mice. Accordingly, a series of experiments was carried out with mammary adenocarcinomas of C3H/He mice. After this work had been completed, a paper by Fardon and Prince (2) appeared describing similar experiments in which mammary carcinoma dbrB was studied in DBA/1 mice.

MATERIALS AND METHODS

C3H (Heston subline) and C57Bl/6 mice, received from the Jackson Memorial Laboratory, and ZBC mice,[1] obtained from the surplus stock of Dr. J. J. Bittner, were used in the experiments. Spontaneous mammary adenocarcinomas 108 and 21 arose in breeding females of the C3H/He strain; they were transplanted at approximately 3-week intervals in C3H/He mice. The Z tumor was obtained from a breeding female of the Z strain from Dr. Bittner; it was carried in ZBC mice. These tumors were of the third to seventh transplant generation at the time the present experiments were begun. Adenocarcinoma E 0771 and C3H-BA, obtained from the Jackson Memorial Laboratory, were used in other experiments; these were carried by transplantation in C57Bl/6 and C3H/He mice, respectively. All the tumors grew progressively in the mice specified, killing the hosts in from 4 to 7 weeks.

[1] ZBC mice are produced by mating mice of the A strain and Dr. Bittner's C3H (called Z strain) to produce F_1 hybrids, and mating F_1 females to Z males.

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These tumors were chosen because they differ in their characteristics of transplantability: Spontaneous adenocarcinomas 108, 21, and adenocarcinoma Z are specific; the former grow progressively upon transplantation only in C3H/He mice and the latter only in Z or Z back cross mice (ZBC). In repeated trials in successive transplant generations, in which the C3H/He tumors were implanted into ZBC mice or in which the Z tumor was implanted into C3H/He mice, there have been no exceptions to this observation. In our experience, adenocarcinoma C3H-BA grows progressively in all C3H/He mice when injected subcutaneously or intradermally as tumor cell suspensions, or when implanted by trocar. It grows progressively in all ZBC mice when implanted by trocar. It was observed that a high rate of tumor regression occurred in ZBC mice which had been given subcutaneous injections of saline suspensions of cells of adenocarcinoma C3H-BA. This occurred following the injection of fresh suspensions or sterile, incubated cell suspensions. Most ZBC mice in which the tumor regressed failed to grow progressive tumors when reimplanted by trocar. Injection of mice with incubated cell suspensions was, therefore, employed in certain experiments as a means of inducing immunity in ZBC mice.

Adenocarcinoma C3H-BA growing in C3H/He mice was removed aseptically and finely minced with scissors. After the addition of a small quantity of saline, the tumor cell suspension was transferred to a sterile glass tissue grinder in which the fragments were mashed by gentle trituration. More saline was then added to make a 10 per cent suspension on a wet weight basis. Mice were injected with 0.2 ml. either of freshly prepared suspensions or those which had been incubated in a water bath at 37° C. for 1.5 hours. Progressive tumors grew in approximately one-third of the ZBC mice injected subcutaneously with the fresh or incubated suspensions. In the remainder, palpable tumors developed and then regressed, or, as happened in some instances, no evidence of tumor growth was detected. Adenocarcinoma 108, Z, or C3H-BA were implanted subcutaneously, either by trocar or by saline suspension of tumor cells, in the right scapular region of C3H/He or ZBC mice.

When the tumors reached 0.75–1.0 cm. in diameter, they were ligated. This was accomplished in lightly etherized mice by firmly, but not tightly, drawing a loop of surgical thread around the base of the tumor made accessible by stretching the skin and tumor outward from the muscles of the back. Eight and 24 hours later, additional progressively tighter loops were placed around the skin pocket in which the tumor was enclosed. This procedure was followed in an attempt to facilitate absorption of products of autolysis from the tumor. Within an hour after ligation, the mass was engorged, and the skin turned to a deep purple color; this condition persisted for 24 hours. Drying of the tissue then began, which progressed until finally (within 4–5 days) the tumor disappeared. In some cases additional loops were necessary on the 3d or 4th day to complete the process. When the tumor invaded

the muscle tissue, as happened in a few cases, it was not possible to stop its progressive growth.

In attempts to induce immunity against the chosen tumors, experimental mice were subjected to various procedures: (*a*) Progressively growing tumors in C3H/He and ZBC mice were destroyed by ligation. (*b*) ZBC mice were injected subcutaneously with incubated cell suspensions of adenocarcinoma C3H-BA and were kept under observation until spontaneous regression of the tumors occurred. (*c*) Saline cell suspensions of C3H-BA were also injected into the tails of C3H/He and ZBC mice, care being taken to miss the tail veins. No tumors developed in the latter. Tumors which developed in C3H/He mice were destroyed by intermittent ligation of the tails above the tumor with soft copper wire (5). (*d*) Fragments of an autolyzed tumor (C3H-BA, growing in a C3H/He mouse) ligated 24 hours previously, were implanted by trocar into other C3H/He mice. None of these developed tumors as a result of this implantation. (*e*) In other experiments, 0.5 ml. of citrated blood obtained by heart puncture from C3H/He mice was injected intraperitoneally, and mammary tissue from nursing C3H/He mice was implanted subcutaneously by trocar into ZBC mice. All the mice were challenged for immunity by grafting fragments of the selected tumors subcutaneously by trocar on the opposite flank. These challenging implantations were made within 2–3 weeks following disappearance of ligated or spontaneously regressed tumors or other preparative treatments to which the mice were subjected. Mice in which the first challenge graft failed to grow were rechallenged after 3 weeks. ZBC mice found to be immune to tumor C3H-BA were rechallenged with Z tumor. All mice were observed for 2 months after this final challenge. Details and results of the experiments are shown in Table 1.

TABLE 1

EFFECT OF PRETREATMENT ON IMMUNITY OF C3H OR ZBC MICE TO REIMPLANTED TUMORS

Tumor	Recipient mouse	Preparatory treatment		No. mice suitable for test of immunity	Immune to reimplanted tumor*					
					C3H-BA		Z		108	
					Yes	No	Yes	No	Yes	No
108	ZBC	Tumor regressed	(24)†	24	23	1	0	23		
"	C3H	Tumor ligated	(20)	15	0	15				
"	"	" "	(28)	28	0	28				
"	"	" "	(20)	20					0	20
C3H-BA	"	" "	(47)	26	0	26				
"	"	" "	(12)	10					0	10
Z	"	Tumor failed to grow	(17)	17	0	17				
"	ZBC	Tumor ligated	(28)	21			0	21		
C3H-BA	"	" "	(54)	41	41	0	0	41		
"	"	Tumor cell suspension injected s.c.	(145)	97‡	89	8	0	55		
"	"	Tumor failed to grow in tail	(10)	10	7	3	0	7		
"	C3H	Tail tumors ligated	(13)	13	0	13				
"	"	Autolyzed tumor implanted	(15)	15	0	15				
	ZBC	Blood from C3H injected	(7)	7	7	0	0	7		
	"	Mammary tissue from C3H injected	(8)	8	8	0	0	8		

* All mice implanted as controls in these tests died of progressive tumors.
† Figures in parentheses indicate number of mice pretreated.
‡ 97 tumors regressed spontaneously.

DISCUSSION

These results are in agreement with those recently described by Fardon and Prince (2), who were unable to induce immunity in DBA/1 mice by ligation of mammary carcinoma dbrB. By the methods used, immunity has not been induced in C3H/He mice against spontaneous mammary adenocarcinoma 108 or adenocarcinoma C3H-BA, or in ZBC mice against a mammary carcinoma which arose in a mouse of Dr. Bittner's Z stock. Similarly, in experiments not recorded in Table 1, no immunity has been observed in C57Bl/6 mice against adenocarcinoma E 0771, or in C3H/He mice against adenocarcinoma 21. However, many ZBC mice were rendered immune to reimplantation of adenocarcinoma C3H-BA, which regularly grows progressively following implantation by trocar. This could be accomplished by a number of procedures including prior injection of blood or mammary tissue from C3H/He mice. Such mice, although immune to adenocarcinoma C3H-BA, were not immune to the adenocarcinoma Z, to which ZBC mice are genetically susceptible. It

would appear that adenocarcinoma C3H-BA is a relatively nonspecific tumor, being transplantable to both C3H/He and ZBC mice, as contrasted with spontaneous tumors 108 and 21, which grow progressively only in C3H/He mice, and spontaneous tumor Z, which grows only in Z or in ZBC mice. In this respect adenocarcinoma C3H-BA resembles lymphosarcoma 6-C3H-ED, which grows progressively in several sublines of C3H mice and also in ZBC mice (3). It is probable, therefore, that the protection afforded ZBC mice against reimplantation of adenocarcinoma C3H-BA has its basis in genetic differences between the tumor and the recipient mice.

A similar explanation applies to the results which have been obtained with C3H mice which can be immunized against lymphosarcoma 6-C3H-ED by a number of procedures (3–5, 16), to those of Gross with mammary tumors in sublines of C3H mice (8), and probably also to those instances in which regression of tumors in rats has been induced by chemotherapeutic agents (1, 17). Another example of the same phenomenon is shown by the studies of Stoerk *et al.* (15), who found that immunity to the Murphy-Sturm lymphosarcoma of rats could be induced by injection of homologous lymphoid cells. Such results indicate that progressive growth of tumors upon transplantation is no assurance of genetic homogeneity between the graft and the susceptible host, and that immunogenetic manifestations may be observed following a variety of experimental procedures when tumors are transplanted into genetically dissimilar hosts or when tumors which have mutated are implanted into inbred animals of the strain of origin.

SUMMARY

Experiments are described in which attempts were made to induce immunity in C3H/He and in ZBC mice by strangulation of implanted strain-specific mammary carcinoma. C3H/He mice in which tumors were thus destroyed were not immune upon reimplantation with adenocarcinoma C3H-BA, nor with two other spontaneous mammary tumors which arose in this strain. Similarly, ZBC mice were not immune upon reimplantation with a mammary tumor which arose in the Z strain, nor were C57Bl/6 mice immune to reimplantation of adenocarcinoma E 0771.

Induced autolysis and destruction of these strain-specific mammary adenocarcinomas growing in mice of the strain in which they arose spontaneously does not lead to immunity against the same strain-specific tumor.

REFERENCES

1. Crossley, M. A.; Allison, J. B.; and Muenzen, J. B. Treatment of Flexner-Jobling Carcinoma in Sprague Dawley Rats with 2,4,6-Triethylenimino-*s*-Triazine. Proc. Soc. Exper. Biol. & Med., **80**:452–55, 1952.
2. Fardon, J. C., and Prince, J. E. An Attempt To Produce Resistance in an Inbred Strain of Mice by Ligation of a Homologous Tumor. Cancer Research, **13**:9–13, 1953.
3. Foley, E. J. Retardation and Regression of Lymphosarcoma 6-C3H-ED in Mice Treated with Alien Mouse Spleen and A-Methopterin. Proc. Soc. Exper. Biol. & Med., **79**:155–58, 1952.
4. ———. Resistance of C3H Mice to Lymphosarcoma 6-C3H-ED Induced by Tissues from Mice of C3H Sublines. *Ibid.*, **80**:671–74, 1952.
5. ———. Immunity of C3H Mice to Lymphosarcoma 6-C3H-ED Following Regression of the Implanted Tumor. *Ibid.*, pp. 675–77.
6. Gross, L. Intradermal Immunization of C3H Mice against a Sarcoma That Originated in an Animal of the Same Line. Cancer Research, **3**:326–33, 1943.
7. ———. The Importance of Dosage on the Intradermal Immunization against Transplantable Neoplasms. *Ibid.*, pp. 770–78.
8. ———. Immunological Relationship of Mammary Carcinomas Developing Spontaneously in Female Mice of a High Tumor Line. J. Immunol., **55**:297–307, 1947.
9. Hauschka, T. S. Immunological Aspects of Cancer. Cancer Research, **12**:615–33, 1952.
10. Lewis, M. R., and Aptekman, M. B. Atrophy of Tumors Caused by Strangulation and Accompanied by Development of Tumor Immunity in Rats. Cancer, **5**:411–13, 1952.
11. Lewis, M. R.; Maxwell, D. B.; and Aptekman, M. B. Atrophy of Sarcoma in Rats Followed by Tumor Immunity. Surgery, **30**:689–94, 1951.
12. MacDowell, E. C.; Taylor, M. J.; and Potter, J. S. Immunization of Mice Naturally Susceptible to a Transplantable Leukemia. Proc. Soc. Exper. Biol. & Med., **32**:84–86, 1934.
13. Snell, G. D.; Cloudman, A. M.; Failor, E.; and Douglas, P. Inhibition and Stimulation of Tumor Homoiotransplants by Prior Injection of Lyophilized Tumor Tissue. J. Nat. Cancer Inst., **6**:303–16, 1946.
14. Spencer, R. R. Tumor Immunity. J. Nat. Cancer Inst., **2**:317–32, 1942.
15. Stoerk, H. S.; Budzilovich, T.; and Bielinski, T. C. Resistance to Grafting with Lymphosarcoma Cells in Rats Injected with Homologous Lymphoid Cells. J. Mt. Sinai Hospital, **29**:169–74, 1952.
16. Stoerk, H. S., and Emerson, G. A. Complete Regression of Lymphosarcoma Implants Following Temporary Induction of Riboflavine Deficiency in Mice. Proc. Soc. Exper. Biol. & Med., **70**:703–4, 1949.
17. Sugiura, K., and Stock, C. C. Studies in a Tumor Spectrum. I. Comparison of the Action of Methylbis(2-chloroethyl)amine and 3-Bis(2-chloroethyl)aminomethyl-4-methoxymethyl - 5-hydroxy - 6-methylpyridine on the Growth of a Variety of Mouse and Rat Tumors. Cancer, **5**:382–402, 1952.
18. Woglum, W. H. Immunity to Transplantable Tumors. Cancer Review, **4**:129–214, 1929.

The Effect of Russian Encephalitis and Other Viruses on Mouse Leukemia*

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The inhibition of the leukocytosis and leukemic infiltration of mouse leukemia by concurrent infection with West Nile and Ilheus viruses has been previously reported (8). There was slight, but probably insignificant, prolongation of survival time in those studies. The present paper reports a similar effect of Russian spring-summer encephalitis virus (R.S.S.E.) and presents results of a survey study of the effects of fourteen other virus infections upon mouse leukemia. The literature concerning antileukemic effects of viruses has been reviewed in a previous publication (8), and the broader field of the oncolytic effects of viruses in experimental animals and man has also been recently reviewed (9, 10).

MATERIALS AND METHODS

The materials and methods were essentially the same as those previously described (8). Ak4 leukemia (in Akm strain mice) was used with all viruses.[1] The leukemic process was initiated by the intraperitoneal inoculation of one hundred thousand or one million cells from a spleen of a leukemic donor mouse. Blood was obtained by nicking a tail vein; blood cell counts were made by routine clinical technics. The leukocyte counts recorded in the tables and figures are the arithmetic means of all ten mice or all survivors in each group. Differential counts were done on only three randomly selected mice per group, and the percentages were converted to absolute counts for tabulation and graphing. Blood counts were performed at intervals of 1–3 days, starting on day 3 or 4 after the injection of leukemia. Each experimental group consisted of 30 or 40 mice, divided into groups of ten for the various treatments, or for untreated leukemic controls. When dead mice were not badly autolyzed, they were autopsied and their liver and spleen weights were recorded.

In choosing the amount of virus to be inoculated and the time of virus inoculation, an attempt was made to have the maximum virus proliferation coincide with the height of the leukemic process. Maximum virus proliferation was assumed to occur on the day of death of the virus controls. It is felt that this schedule provided the greatest possibility of suppression of the leukemic process, although it is apparent that this time schedule would obscure possible effects on survival time because the time of death from virus infection would coincide with the time of death of the leukemic controls. At least two groups of ten leukemic mice were given inoculations of each virus, usually on day 4 and on day 6 or 7 after injection of leukemia, in an attempt to provide the maximum possibility of demonstrating an antileukemic effect. Even so, the desired time relationship of virus propagation to leukemic cell propagation was undoubtedly missed in many experiments, especially with those viruses such as Bunyamwera which characteristically kill mice within 3–5 days after inoculation. In the experiments with R.S.S.E. the time of virus inoculation was varied from 2 days before to 7 days after injection of the leukemia to determine the time limits within which the antileukemic effect of this virus might be demonstrated.

The virus inocula were infected mouse brain suspensions, except that the Newcastle disease virus (NDV) preparations consisted of infected allantoic fluid from 10-day chick embryos, and the vaccinia preparations were from chorioallantoic membrane of 14-day chick embryos. All virus preparations were titrated to prove the presence of viable virus by an appropriate established technic for each virus. In addition, in each experiment, groups of five Swiss (Banks) mice were given inoculations of the same dose of virus by the same route as the leukemic experimental mice. Some or all of these virus controls died in each experiment (with the exception of those given neurovaccinia and dermo-vaccinia in some experiments, and N.D.V. in all experiments) at a time and in a manner consistent with the virus inoculated. The virus was inoculated intraperitoneally (I.P.), except for those viruses with which infection could not be established by that route. In these cases (see Table 1) the virus was given intracerebrally (I.C.).

RESULTS AND DISCUSSION

It was previously demonstrated that injection of normal mouse brain had no effect upon the leukemic process and that West Nile and Ilheus viruses did not affect the blood picture of non-leukemic mice (8). In the present study, it was demonstrated that normal allantoic fluid (I.C. or I.P.) had no antileukemic effect. The effects of the present group of viruses upon the blood picture of normal mice have not been studied.

The fifteen viruses studied can be divided into three groups on the basis of their effect upon the leukemic process (Table 1): (*a*) Viruses with

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clearly established antileukemic effect.—The only virus with such capacity was R.S.S.E. (in addition to the previously studied West Nile and Ilheus viruses). (*b*) Viruses which, in this study, had no detectable antileukemic effect.—Some of the viruses included in this category were not tested under optimal conditions for the demonstration of antileukemic effects. This reservation applies to those viruses which were administered intracerebrally (under these conditions systemic distribution of the virus might not occur), those which were inoculated in sublethal doses (since it is not known whether such viruses were able to propagate at all), and those which were administered at times which did not permit maximal virus effects to coincide with maximum development of the leukemia. (*c*) Viruses with slight or questionable antileukemic effect.—This category includes viruses which in some experiments appeared to inhibit the leukemia but which in repeated studies did not, and other viruses which inhibited the leukemic leukocytosis only slightly or very briefly. Table 1 also contains key references concerning the source and properties of each virus.

TABLE 1

LEUKEMIA-SUPPRESSING CAPACITY OF VIRUSES AGAINST AK4 LEUKEMIA OF MICE

Inhibitory	Slight or inconsistent inhibition	No inhibition
Russian spring-summer encephalitis (6, 11)	Bunyamwera (6, 11)	Zika (2)
West Nile* (6, 11)	Uganda S† (1)	Semliki (6, 11)
Ilheus* (6, 11)	Anopheles B† (6, 11)	M. M. (Encephalomyocarditis) (6)
	N.D.V. (Mass.)‡	Neuroflu (3)
	Theiler's G D VII (11)	Dermo Vaccinia‡ (11)
		Neurovaccinia (11)
		N.D.V. (C G 179)†‡ (4)
		Mac Dowell (5)
		Ntaya (7)

* Previously reported (8). West Nile virus was restudied 3 times (three groups of ten mice, virus given I.C. to one group) and Ilheus once in the present experiments, and the previously reported effects were confirmed. The Egypt 101 isolate of West Nile virus was also studied and showed slight to definite antileukemic effects by both I.C. and I.P. routes.

† Studied intracerebrally only.

‡ Virus was not lethal for the nonleukemic virus controls (Swiss mice, Banks).

The failure of "Mac Dowell virus" to affect Ak4 leukemia deserves mention, because this virus was discovered as a contaminant in line I leukemia of C57 mice; in this situation it did have a slight antileukemic effect. Failure to duplicate this effect in the present studies may be owing to our use of a different strain of mice and of leukemia, or, possibly, because our test system is less sensitive than that used by Mac Dowell *et al.* (5). The other viruses which were without antileukemic effect in these experiments will not be discussed further.

The mice inoculated with G. D. VII (Theiler's) virus on day 4 after injection of the leukemia showed a transient inhibition of the leukemic leukocytosis on days 7 and 8 but not thereafter. The total leukocyte counts were 19,200 and 46,800 cells per cubic millimeter on days 7 and 8, respec-

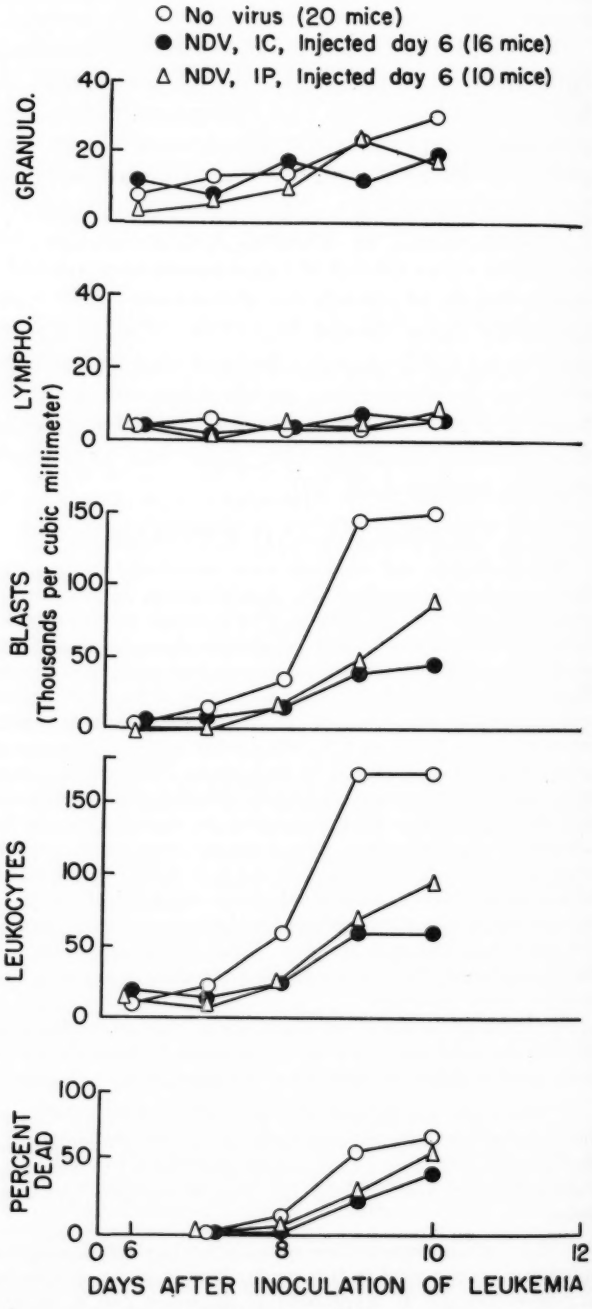

CHART 1.—The effects of Newcastle disease virus (? noninfective) on survival and blood picture of mice bearing Ak4 leukemia.

tively, in the virus-treated group, as compared to 45,900 and 89,100, respectively, in the parallel untreated controls. Survival curves were essentially equal for the two groups. When G. D. VII was

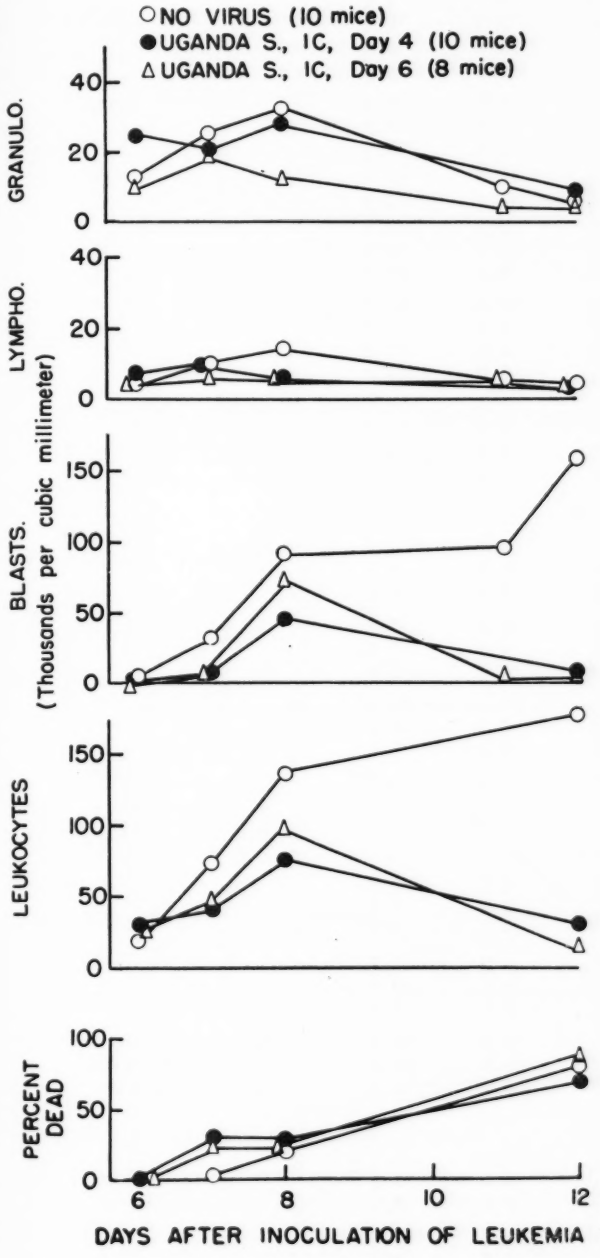
○ NO VIRUS (10 mice)
● UGANDA S., IC, Day 4 (10 mice)
Δ UGANDA S., IC, Day 6 (8 mice)
GRANULO.
LYMPHO.
BLASTS. (Thousands per cubic millimeter)
LEUKOCYTES
PERCENT DEAD
DAYS AFTER INOCULATION OF LEUKEMIA

CHART 2.—The effects of Uganda S virus infection on survival and blood picture of mice bearing Ak4 leukemia.

inoculated on day 1, no influence on the leukocytosis was detected. In one experiment in which Bunyamwera virus was inoculated on day 4 after infection with leukemia, the total leukocyte counts for the virus-treated group were 7,400, 11,400, and 44,700 on days 6, 7, and 8, respectively, as compared to 16,300, 44,200, and 64,700 in the parallel controls. Survival curves were equal for the two groups. In two other experiments with Bunyamwera, the virus killed the mice so rapidly that valid comparison with the control groups was impossible.

Data concerning three other viruses (Newcastle disease, Uganda S, and Anopheles B), which showed a slight or inconsistent antileukemic effect, are presented in Charts 1–3. The fact that NDV (Massachusetts strain) had a slight antileukemic effect is of interest, because this virus is pathogenic for fowl, but not for mice. However, the antileukemic effect was not increased by the

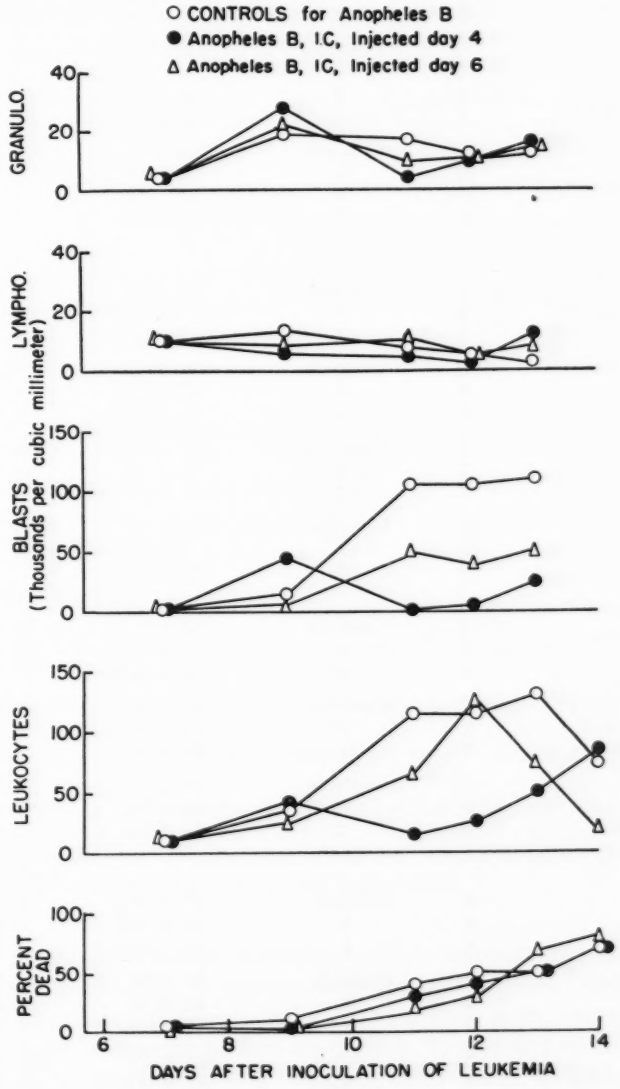

CHART 3.—The effects of Anopheles B virus infection on survival and blood picture of mice bearing Ak4 leukemia.

administration of multiple doses of the virus (0.5 cc. undiluted infected allantoic fluid, I.P. twice daily for six doses, starting 4 days after injection of leukemic cells).

The inhibition of leukemic leukocytosis by

TABLE 2

EFFECT OF I.P. INOCULATION OF R.S.S.E. VIRUS ON AK4 LEUKEMIA

Days after leukemia inoculation	Days after virus inoculation	Total leukocytes*	Blasts*	Lympho-cytes	Granulo-cytes	Survival† Av.	Survival† Range (days)
			Experiment Z				
4	Controls	14.5 (10)	1.4 (3)	4.7	6.2		
4	4	12.8 (10)	1.3 (3)	5.1	6.1		
4	2	18.9 (10)	1.6 (3)	7.3	8.7		
4	0	20.0 (10)	2.3 (3)	11.2	14.1		
7	Controls	59.7 (10)	34.0 (3)	7.7	37.5		
7	7	14.6 (10)	2.4 (3)	4.2	7.2		
7	5	22.2 (10)	3.2 (3)	5.3	17.2		
7	0	78.6 (10)	21.4 (3)	11.0	29.5		
8	Controls	187.3 (9)	197.0 (3)	15.6	34.1		
8	8	13.8 (6)	1.5 (3)	2.7	6.8		
8	6	50.0 (10)	16.5 (3)	10.1	29.9		
8	1	110.4 (4)	108.1 (3)	9.4	26.5		
9	Controls	148.8 (7)	136.8 (3)	11.8	21.7		
9	9	12.5 (3)	2.1 (3)	3.2	7.2		
9	7	56.1 (7)	35.1 (3)	5.3	11.9		
9	2	48.0 (3)	28.5 (3)	6.0	10.1		
10	Controls	192.2 (2)	179.5 (2)	10.5	7.6	10.1	(8–14)
10	10	4.8 (1)	1.2 (1)	1.7	1.9	9.0	(8–11)
10	8	10.5 (2)	2.9 (2)	3.2	4.5	10.2	(9–14)
10	3	14.1 (2)	7.4 (2)	0.8	5.4	9.2	(8–14)
			Experiment W				
5	Controls	12.0 (9)	2.4 (3)	2.5	4.9		
5	7	10.2 (10)	2.2 (3)	3.3	4.9		
5	5	10.6 (10)	1.4 (3)	6.4	3.5		
6	Controls	38.7 (8)	13.0 (3)	13.3	13.2		
6	8	20.3 (7)	4.7 (3)	4.0	7.0		
6	6	11.8 (10)	1.8 (3)	2.2	6.9		
7	Controls	108.2 (6)	75.7 (3)	12.1	30.2		
7	9	38.4 (3)	11.3 (3)	6.4	14.5		
7	7	11.6 (9)	1.2 (3)	2.3	5.6		
10	Controls	215.6 (4)	127.4 (1)	8.3	2.8	10.3	(6–12)
10	12	(0)				7.1	(6–9)
10	10	55.0 (1)				9.8	(6–11)
			Experiment V				
6	Controls	15.8 (10)	2.9 (3)	9.7	14.2		
6	2	10.4 (9)	1.5 (3)	3.5	9.3		
6	0	21.0 (10)	7.0 (3)	3.7	8.4		
7	Controls	45.1 (8)	43.2 (3)	8.0	27.7		
7	3	28.5 (9)	2.0 (3)	4.6	10.8		
7	1	38.3 (8)	2.2 (3)	5.8	5.3		
8	Controls	97.1 (6)	76.0 (3)	9.1	15.0	9.5	(6–16)
8	4	32.6 (6)	21.3 (3)	9.7	15.8	8.7	(5–11)
8	2	34.6 (6)	13.2 (3)	6.0	12.6	9.8	(6–13)
			Experiment U				
6	Controls	17.2 (10)	8.5 (3)	5.0	8.1		
6	2	18.5 (10)	2.2 (3)	4.5	7.3		
6	0	16.1 (10)	2.7 (3)	8.0	6.1		
7	Controls	50.4 (6)	43.7 (3)	14.0	18.8		
7	3	44.9 (8)	26.0 (3)	6.1	19.1		
7	1	36.2 (5)	9.1 (3)	7.3	9.2		
8	Controls	262.0 (2)	224.3 (2)	3.1	34.7		
8	4	42.9 (8)	23.9 (3)	2.8	16.3		
8	2	113.5 (2)	81.9 (2)	10.2	21.4		
11	Controls	(0)				7.9	(7–10)
11	7	28.8 (1)				9.5	(7–11)
11	5	22.5 (1)				8.3	(7–14)

* All blood counts are expressed as thousands of cells per cubic millimeter of blood. The figures in parentheses following the mean blood cell counts indicate the number of mice averaged, and in the leukocyte column this figure also indicates the numbers of mice surviving on that day. Since differential counts were not done on all mice, the sum of the average differential counts is not identical with the average total leukocyte counts.

† Survival times are computed from the inoculation of leukemia to death, regardless of the time of virus inoculation.

R.S.S.E. virus is indicated by the data presented in Table 2 and Chart 4. In the graph, data from comparable time schedules of various experiments

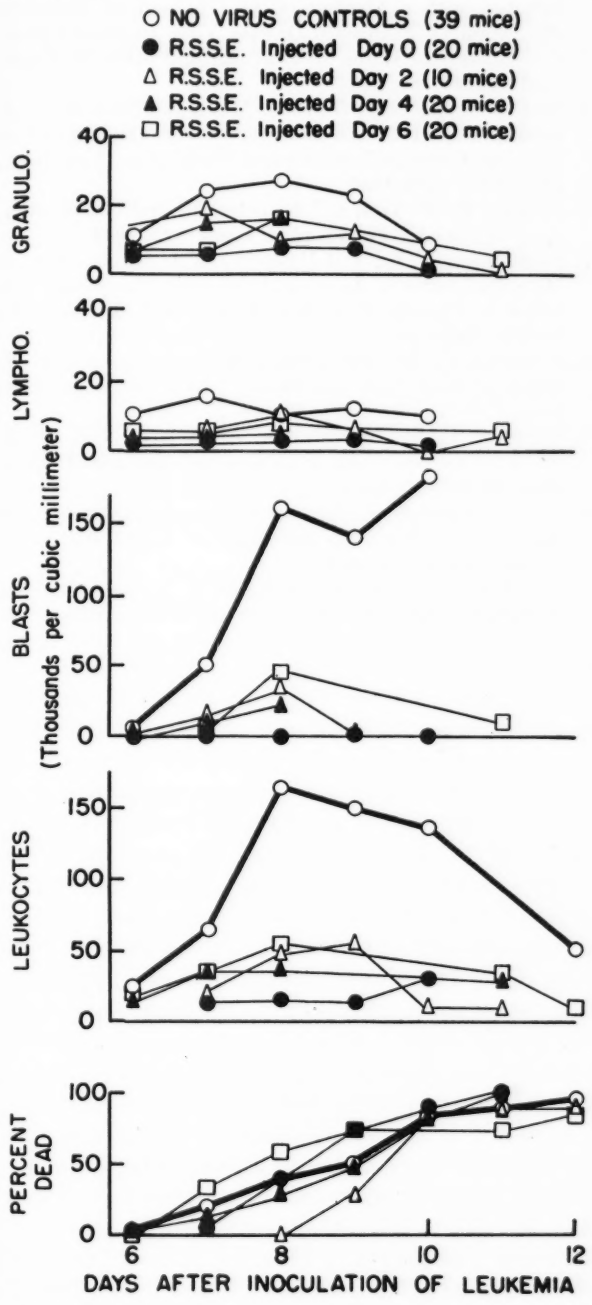
○ NO VIRUS CONTROLS (39 mice)
● R.S.S.E. Injected Day 0 (20 mice)
△ R.S.S.E. Injected Day 2 (10 mice)
▲ R.S.S.E. Injected Day 4 (20 mice)
□ R.S.S.E. Injected Day 6 (20 mice)
GRANULO.
LYMPHO.
BLASTS
(Thousands per cubic millimeter)
LEUKOCYTES
PERCENT DEAD
DAYS AFTER INOCULATION OF LEUKEMIA

CHART 4.—The effects of Russian spring-summer encephalitis virus infection on survival and blood picture of mice bearing Ak4 leukemia.

are averaged and presented as a single curve. In the table, data from each experiment are averaged separately to permit more valid comparisons of treated and control groups. The leukocytosis was retarded even when the virus was inoculated after the leukocyte count had started to rise (see experiment Z, Table 2). This suppressive effect was due to selective inhibition of the emergence of blast forms, as judged by differential leukocyte counts.

The antileukemic effect of some of these viruses was also suggested by the smaller size and weight of livers and spleens of virus-treated mice at autopsy when compared to the parallel, untreated controls. These data from the blood-count experiments were too scattered, however, to permit valid comparisons, so one experiment was designed to permit a more adequate investigation of this phenomenon. Fifteen mice were injected with

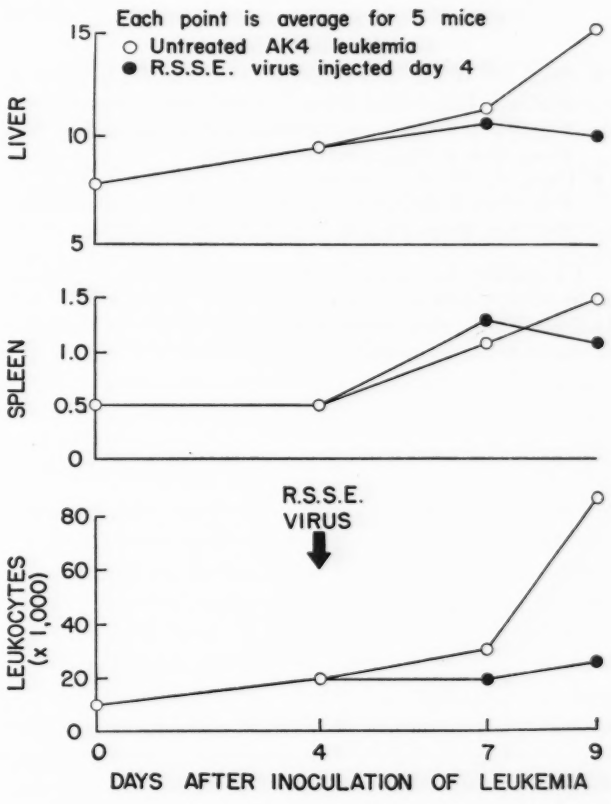

CHART 5.—The effects of Russian spring-summer encephalitis virus infection on the leukocytosis, splenomegaly, and hepatomegaly of Ak4 mouse leukemia. Organ weights are expressed on percentage of total body weight.

R.S.S.E. virus on the 4th day after transplantation of leukemia. Virus-treated leukemic mice and parallel untreated leukemic controls were sacrificed in groups of five on days 4, 7, and 9. Leukocyte counts, spleen weights, and liver weights were determined at the time of sacrifice. Data from this experiment are presented in Chart 5. Nine days after inoculation of leukemia and 5 days after inoculation of the virus, the weight of livers in untreated mice had doubled (owing to leukemic infiltration), whereas there was only a 33 per cent increase in the size of the liver in virus-infected mice. Spleen weights showed the same type of

effect, although it was less marked.[2] Leukocytosis was also markedly inhibited.

SUMMARY

R.S.S.E. virus has been demonstrated to inhibit leukocytosis and organ infiltration in Ak4 mouse leukemia. Survival time was not affected. The Massachusetts strain of N.D.V., Uganda S virus, Anopheles B virus, Bunyamwera virus, and Theiler's G.D. VII virus had slight antileukemic effect. Nine other strains of viruses had no detected effect upon this leukemia.

REFERENCES

1. Dick, G. W. A., and Haddow, A. J. Uganda S Virus. A Hitherto Unrecorded Virus Isolated from Mosquitoes in Uganda. I. Isolation and Pathogenicity. Trans. Roy. Soc. Trop. Med. & Hyg., **46**:600–618, 1952.
2. Dick, G. W. A.; Kitchen, S. F.; and Haddow, A. J. Zika Virus. I. Isolations and Serological Specificity. Trans. Roy. Soc. Trop. Med. & Hyg., **46**:509–20, 1952.
3. Francis, T., and Moore, A. E. A Study of the Neurotropic Tendency in Strains of Virus of Epidermic Influenza. J. Exper. Med., **72**:717–28, 1940.
4. Karzon, D. T., and Bang, F. B. The Pathogenesis of Infection with a Virulent (C G 179) and an Avirulent (B) Strain of Newcastle Disease Virus in the Chicken. I. Comparative Rates of Viral Multiplication. J. Exper. Med., **93**:267–84, 1951.
5. Mac Dowell, E. C.; Taylor, M. J.; and Broadfoot, J. Annual Report of the Director of the Department of Genetics, Carnegie Institution of Washington. Year Book, **49**:178–81, 1949–1950.
6. Rivers, T. M. Viral and Rickettsial Infections of Man, pp. 719, (2d ed.). Philadelphia: Lippincott, 1952.
7. Smithburn, K. C., and Haddow, A. J. Ntaya Virus. A Hitherto Unknown Agent Isolated from Mosquitoes Collected in Uganda. Proc. Soc. Exper. Biol. & Med., **77**: 130–33, 1951.
8. Southam, C. M.; Bronstein, B.; and Webber, L. F. Effect of West Nile and Ilheus Viruses on Mouse Leukemias. Cancer Research, **11**:669–75, 1951.
9. Southam, C. M., and Moore, A. E. West Nile, Ilheus, and Bunyamwera Virus Infections in Man. Am. J. Trop. Med., **31**:724–41, 1951.
10. ———. Clinical Studies of Viruses as Antineoplastic Agents, with Particular Reference to Egypt 101 Virus. Cancer, **5**:1025–34, 1952.
11. Van Rooyen, C. E., and Rhodes, A. J. Virus Diseases of Man, pp. 1202. New York: Nelson & Sons, 1948.

[2] A similar observation of decreased leukemic infiltration of livers and spleens of Ak4 and Ak9421 leukemic mice following R.S.S.E. virus infection was made by Dr. A. E. Moore of this institute (unpublished data).

Autoradiography of Mast Cells in Experimental Skin Tumors of Mice Injected with Radioactive Sulfur (S^{35})*

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Mast cells are present in the organism wherever there is connective tissue. They are large, with cytoplasmic granules which stain metachromatically with certain basic dyes, such as toluidine blue. Recent research on connective tissue has clarified the chemistry and thus the function of these cells.

INTRODUCTION

In 1937, Holmgren and Wilander (10) maintained that mast cells in connective tissue contain a polysulfuric acid ester giving a heparin effect and that it is their function to secrete the anticoagulative substance into the blood. This contention is supported by the finding that tissue which is rich in mast cells—e.g., the hepatic capsule of the cow—has more pronounced anticoagulative properties than other tissue with a lower content of mast cells—such as the hepatic capsule of the sheep. Moreover, these authors based their view on Lison's (12) opinion that metachromatic staining with toluidine blue is due to the presence of high molecular polysulfuric acid esters. Heparin is one such substance, a sulfuric mucopolysaccharide. Subsequent workers, however, have shown that other substances, such as monosulfuric acid esters and the nonsulfuric but closely related mucopolysaccharide hyaluronic acid (which makes up a considerable part of the connective tissue ground substance) stain metachromatically with toluidine blue and similar dyes (1–5, 7, 14, 16, 18). Like heparin, hyaluronic acid is a polymer which, on hydrolysis, gives glucosamine and glucuronic acid; but unlike heparin it does not contain sulfur. The mucopolysaccharide in the cornea, a monosulfuric acid ester of hyaluronic acid, stains metachromatically.

In 1948, Jorpes, Werner, and Åberg (11) found that, besides staining metachromatically like the acid mucopolysaccharides, mast-cell granules stain with leucofuchsin following oxidation with periodic acid by the method of McManus (13). They pointed out that heparin and chondroitin sulfuric acid do not stain this way, whereas hyaluronic acid and mucoitin monosulfuric acid are stained distinctly. Jorpes and co-workers concluded that the compound present in the mast-cell granules, although not heparin proper, may be its precursor.

Asboe-Hansen (2–5) was unable to demonstrate a definite relation between the number of mast cells in the tissue and its content of sulfomucopolysaccharides, whereas he found a correlation between the number of mast cells and the quantity of hyaluronic acid in the tissue, as determined by chemical and histochemical methods. The author also observed signs of a new formation of hyaluronic acid from mast cells following injection of hyaluronidase into the tissue (*in vivo* experiments). Apparently, under the influence of hormonal action, the mast cells secrete the mesenchymal mucopolysaccharide, hyaluronic acid, perhaps by way of a sulfuric precursor resembling heparin.

It is known (6, 17) that when a number of high molecular nonsulfuric polysaccharides, including hyaluronic acid, are sulfonated they may acquire anticoagulative properties.

From chemical, histochemical, and histophysiological investigations it seems justifiable to infer that the mast cells contain a sulfuric mucopolysaccharide closely related to heparin and hyaluronic acid, though not identical with either, however. The mast cells seem to give off a hyaluronidase-sensitive mucopolysaccharide to the connective-tissue ground substance, which does not appear to possess anticoagulative properties.

The chemical changes in the substance of the granules possibly occurring before, during, or after its transfer from the cell to the connective tissue ground substance are unknown and the subject of intense study in various laboratories.

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MATERIALS AND METHODS

Autoradiographic investigation.—Histochemical methods cannot offer quantitative information, and chemical analysis of tissue reveals nothing concerning the location of sulfur in the tissue. The author therefore used autoradiography to demonstrate the localization of sulfur-containing compounds in connective tissue, their relative quantities and distribution.

In order to obtain connective tissue containing mast cells in large numbers, experimental skin tumors (papillomas), containing cells presumably at the maximum of their functional capacity, were induced in mice of the strain St/Eh by a single painting with 9,10-dimethyl-1,2-benzanthracene, a carcinogenic hydrocarbon. In the dermal connective tissue of the painted areas the tumor-bearing mice revealed a marked accumulation of mast cells, most of which are large and richly granulated (8, 9). Nine of these tumor-bearing mice received one intraperitoneal injection of S^{35}, 8 μc/gm of body weight with 0.1 mg. Na_2SO_4 as carrier in 0.2 ml. of sterile water. After 48 hours the mice were killed; the tumor and a piece of normal skin from a symmetrical site were excised and divided into three portions, which were fixed in 70 per cent alcohol, 10 per cent formalin, or 4 per cent basic lead acetate, respectively, while one portion was freeze-dried. The tissue was imbedded in paraffin and cut in sections 7 μ thick. After the sections were deparaffinized and dried, autoradiographs were made applying the stripping film technic developed by Pelc (15). The films were exposed for 17 days. Some unstained preparations and the superimposed autoradiographs were studied in the phase contrast microscope; others were stained, after exposure, with ½ per cent aqueous solution of toluidine blue for 15 minutes and then inspected microscopically.

To check the autoradiographic method as applied here, a number of controls were made on mice which had not been injected with S^{35}. Tissue sections from tumor and from normal skin did not cause blackening of the stripping film after several days' exposure. It can therefore be concluded that the results obtained by autoradiography after injection of S^{35} are not due to chemography or some other artefact inherent in the histological preparation proper.

RESULTS AND CONCLUSIONS

Forty-eight hours after injection of radioactive sulfur, monitoring with a Geiger counter revealed a pronounced accumulation of radiosulfur in the tumor.

After 17 days' exposure, a stripping film showed marked blackening over the mast cells in the connective tissue of the skin tumor. In addition, there was a less intense, diffuse blackening over the connective tissue ground substance. In autoradiographs of normal, unpainted skin, only a slight blackening could be observed.

A few mast cells in the connective tissue of the unpainted skin and in muscle gave no distinct blackening, and in some of them the content of radiosulfur could not be demonstrated with certainty.

It cannot be ascertained whether the diffuse blackening of the film, produced by the connective tissue in the tumor, is due to radiosulfur leached out from the mast cells in the course of the histological fixation or during the stripping procedure, or whether it is caused by an increased content of sulfur in the connective tissue ground substance of the tumor. The autoradiographs were very similar, independent of whether the specimens were fixed in one of the three different solutions or freeze-dried. Furthermore, the blackening caused by mast cells appears to be very much the same in all preparations. This is noteworthy in view of the fact that in histological preparations mast cells appear larger and more regular in freeze-dried specimens and after fixation with basic lead acetate than after fixation in alcohol.

Whether the ability of mast cells to take up radiosulfur depends on their age and metabolic state, whether the experimental period (48 hours) is too long or too short, whether or not the sulfuric acid content of individual mast cells varies—these are problems which require extensive investigations. Such studies are in progress.

SUMMARY

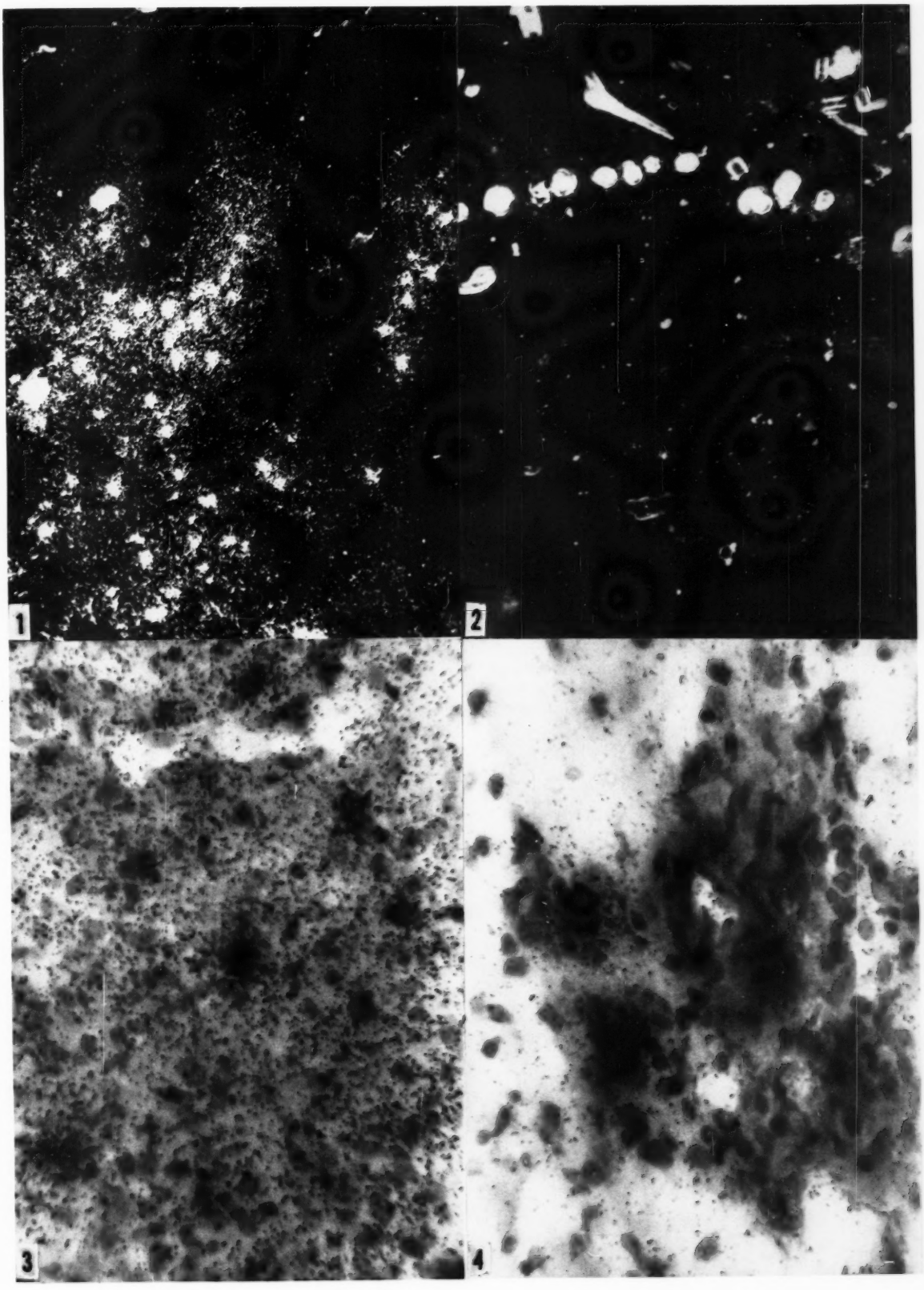
Previous histochemical and chemical studies indicated that the granules of the connective tissue

FIG. 1.—Autoradiograph from section of experimental skin tumor; unstained preparation; dark-field microscopy. Above, the hyperplastic epithelium with deep extensions into the corium. In the connective tissue the mast cells appear as bright discs, indicating a high radiosulfur content; diffuse blackening over the ground substance. Mag. ×350.

FIG. 2.—Unpainted skin from a site symmetrical to the skin area in Fig. 1; unstained; dark-field microscopy; radiosulfur content low. Hairs above. Mag. ×350.

FIG. 3.—Unstained preparation; connective tissue area of Fig. 1. Eleven mast cells have caused intense blackening of the stripping film. Mag. ×1200.

FIG. 4.—Stained preparation. Staining: toluidine blue, ½ per cent aqueous solution, for 15 minutes. A vessel with two mast cells in its immediate surroundings. Intense blackening of the film over the sites of the mast cells. Mag. ×1350.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mast cells contain a sulfuric mucopolysaccharide. This substance is closely related to heparin and hyaluronic acid without being identical with either.

Stripping-film autoradiography of connective tissue in experimental skin tumors in mice injected intraperitoneally with S^{35}, with sodium sulfate as carrier, showed that the majority of the mast cells take up sulfur. This uptake manifests itself as a blackening of a stripping film.

ACKNOWLEDGMENTS

The author wishes to express his indebtedness to Hilde Levi, Ph.D., from the Department of Biological Isotope Research, University of Copenhagen, for valuable guidance in the work with radioactive tracers and in the application of autoradiography. The autoradiographs mentioned in the present paper were made in Dr. Levi's laboratory.

REFERENCES

1. ALTSCHULER, C. H., and ANGEVINE, D. M. Histochemical Studies on the Pathogenesis of Fibrinoid. Am. J. Path., **25**:1061–75, 1949.
2. ASBOE-HANSEN, G. The Origin of Synovial Mucin. Ehrlich's Mast Cell—a Secretory Element of the Connective Tissue. Ann. Rheumat. Dis., **9**:149–58, 1950.
3. ———. The Variability of the Hyaluronic Acid Content of the Dermal Connective Tissue under the Influence of Thyroid Hormone. Acta Dermat-venereol., **30**:221–30, 1950.
4. ———. La "Mastzelle," facteur important dans la formation de la substance fondamentale conjonctive et de la synovie. Bull. d'Histol., **27**:5–11, 1950.
5. ———. Om Bindevævets Mucinøse Substanser. (On the Mucinous Substances of the Connective Tissue. English Summary.) Copenhagen: Rosenkilde & Bagger, 1951.
6. BALAZS, E. A.; HÖGBERG, B.; and LAURENT, T. C. The Biological Activity of Hyaluron Sulfuric Acid. Acta Physiol. Scandinav., **23**:168–78, 1951.
7. BUNTING, H. The Distribution of Acid Mucopolysaccharides in Mammalian Tissues as Revealed by Histochemical Methods. Ann. New York Acad. Sc., **52**:977–82, 1950.
8. CRAMER, W., and SIMPSON, W. L. Mast Cells in Experimental Skin Carcinogenesis. Cancer Research, **4**:601–16, 1944.
9. ENGELBRETH-HOLM, J., and ASBOE-HANSEN, G. Effect of Cortisone on Experimental Skin Carcinogenesis in Mice. Acta Pathol. et Microbiol. Scandinav., **32**:560–64, 1953.
10. HOLMGREN, H., and WILANDER, O. Beitrag zur Kenntnis der Chemie und Funktion der Ehrlichschen Mastzellen. Ztschr. f. mikr.-anat. Forsch., **42**:242–78, 1937.
11. JORPES, E.; WERNER, B.; and ÅBERG, B. The Fuchsin-Sulphurous Acid Test after Periodate Oxidation of Heparin and Allied Polysaccharides. J. Biol. Chem., **176**:277–82, 1948.
12. Lison, L. Études sur la métachromasie. Arch. Biol., Paris, **46**:599–668, 1935.
13. MCMANUS, J. F. A. Histological Demonstration of Mucin after Periodic Acid. Nature, **158**:202, 1946.
14. MEYER, K. The Biological Significance of Hyaluronic Acid. Physiol. Rev., **27**:335–59, 1947.
15. PELC, S. R. Autoradiograph Technique. Nature, **160**: 749–50, 1947.
16. SYLVÉN, B., and MALMGREN, H. On the Alleged Metachromasia of Hyaluronic Acid. Lab. Invest., **1**:413–31, 1952.
17. WALTON, K. W. The Biological Behaviour of a New Synthetic Anticoagulant (Dextran Sulphate) Possessing Heparin-like Properties. Brit. J. Pharmacol., **7**:370–91, 1952.
18. WISLOCKI, G. B.; BUNTING, H.; and DEMPSEY, E. W. Metachromasia in Mammalian Tissues and Its Relationship to Mucopolysaccharides. Am. J. Anat., **81**:1–31, 1947.

A Comparative Study of Certain Properties of Liver β-Glucuronidase of Inbred Mice*†

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Interest in the genetic factors controlling tissue β-glucuronidase in inbred mice began with the original report of Morrow *et al.* (10), who found that C3H mice exhibited markedly reduced liver β-glucuronidase activity. This phenomenon was explained by Law *et al.* (9) in studies of the inheritance of "low" and "high" tissue glucuronidase tendencies. These showed the existence of a single gene pair which was determining tissue glucuronidase activity for C3H and strain A mice.

Hormonal stimuli have been found to alter tissue β-glucuronidase activity. For example, the administration of testosterone propionate to inbred mice of either sex produced a marked rise in kidney β-glucuronidase activity (4, 5), and stilbestrol enhanced liver β-glucuronidase (5). The extent of the response appeared to be strain-dependent. It was later found (11) that the kidney enzyme response was paralleled by the excretion of β-glucuronidase in the urine.

The present paper deals with a comparison of some of the usually determined enzymic constants of β-glucuronidase preparations made from tissues of groups of low- and high-glucuronidase strains of mice treated and untreated with sex hormones. Similar data have been collected with β-glucuronidase preparations of Ehrlich's ascites tumor and of livers of white Swiss mice. These experiments were designed to provide a partial answer to the question: Is the same β-glucuronidase present in a tissue of "high"- and "low"-glucuronidase strains of mice?

MATERIALS AND METHODS

Adult mice of different strains (Jackson Memorial Laboratory, Bar Harbor, Maine) were used. To study the effect of testosterone propionate and stilbestrol, 0.5 mg/day of the hormone under investigation was injected for 14 days prior to sacrificing the animals (5).

An active extract of β-glucuronidase from each tissue to be studied was prepared by the method of Fishman and Talalay (7). The β-glucuronidase determinations were performed by the method of Fishman *et al.* (5, 6). The pH optimum and the Michaelis constant (K_m) were determined in each case, according to previously described methods (3). In addition, inhibition by potassium acid saccharate and by potassium glucuronate (8), temperature coefficient, presence in the enzyme extract of activating or inhibiting substances, and activation by DNA (1) were investigated for each enzyme preparation. To study the activating effect by DNA, the enzyme was further purified by precipitating it at room temperature with an equal volume of cold saturated ammonium sulfate solution of pH 8.7 and dissolving the residue in H_2O (2).

RESULTS

All data are tabulated in Table 1. There were no noticeable differences of pH optimum of β-glucuronidase prepared from different strains of mice. No second peak of lesser magnitude was observed in the C3H liver preparation at pH 5.2, as described by Morrow (10).

The Michaelis constant observed in the enzymatic hydrolysis of phenolphthalein glucuronide by β-glucuronidase for different preparations varies from 0.000038 to 0.000054, which is in a range similar to the constant previously reported (12).

Potassium acid saccharate exhibited a marked inhibitory action of β-glucuronidase at concentrations much less than those with potassium glucuronate. All dilute enzyme preparations were activated by the presence of 0.3 per cent DNA to the same extent as reported previously (1). The addition of the various enzyme extracts in small amounts (0.1 ml.) to a highly purified β-glucuronidase preparation (activity 4 units/ml) did not alter the enzyme activity measured during a 1-hour period.

Temperature coefficients ranging between 1.5 and 1.9 were observed for the range 30°–40° for different preparations. A decrease of Q_{10} to 1.3–1.7 was observed for the temperature range 50°–60°.

These data indicate the similarity of the liver

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β-glucuronidase prepared from different strains of mice.

DISCUSSION

It is now recognized that certain strains of mice —e.g., AKR (5) C3H (10)—possess markedly reduced tissue β-glucuronidase activities, as compared to other pure (129, DBA) strains. The evidence at present points to a genetic control of this tissue enzyme in mice. It is necessary to establish whether or not these differences in activity are properties of β-glucuronidase itself or merely represent an effect secondary to some nonspecific factor. There is the same need for clarification of the nature of hormone-stimulated β-glucuronidase in the liver and kidney and in β-glucuronidase-rich tumors.

From the data presented, no important differences in the properties of these various β-glucuronidase preparations have been detected. Certainly with respect to the hydrolysis of phenolphthalein β-glucuronide, the same β-glucuronidase appears to be present in tissues of "high" and "low" β-glucuronidase strains of mice. However, the possibility cannot be excluded that real dissimilarities may exist, although this now appears remote.

SUMMARY

Partially purified β-glucuronidase preparations have been made in a uniform manner from livers of inbred mice of the 129, DBA, AKR, and C3H strains and from livers of white Swiss mice. In addition, similar extracts were prepared from livers of stilbestrol-treated AKR mice, from kidneys of testosterone propionate-treated A Cloudman mice, and from cell suspensions of the Ehrlich ascites tumor. A number of properties of β-glucuronidase were examined in these extracts, such as optimum pH, Michaelis const. (K_m), inhibition by saccharate and glucuronate, activation by DNA, temperature coefficient, etc. Within experimental limits, the various enzyme preparations exhibited the same properties. It may be tentatively stated, therefore, that the marked strain differences in tissue β-glucuronidase activity are referable to differences in the amount of enzyme protein.

TABLE 1

PROPERTIES OF β-GLUCURONIDASE PREPARED FROM VARIOUS TISSUES

	Tissues*							
			Liver					
			"low" strains		"high" strains			
Properties	Erhlich ascites tumor cell	White Swiss	AKR	C3H	129	DBA	Stilbestrol-treated AKR	Kidney Testosterone propionate-treated A Cloudman
Range of pH optimum†	4.2–4.6	3.9–4.5	3.8–4.6	4.2–4.5	3.9–4.5	3.6–4.5	3.6–4.7	3.9–4.5
Michaelis constant (K_m)	0.000042	0.000038	0.000054	0.000050	0.000048	0.000046	0.000047	0.000053
Per cent inhibition by 5×10^{-5}M K-acid saccharate	43	45	52	43	45	44	49	37
Per cent inhibition by 2.5×10^{-2}M K-glucuronate	45	43	54	42	52	47	48	56
Temperature coefficient								
20°–30°	1.4	2.3	1.7	1.4	2.0	1.6	2.0	1.7
30°–40°	1.6	1.7	1.6	1.9	1.9	1.5	1.6	1.7
40°–50°	1.6	1.5	1.6	1.9	1.6	1.6	1.5	1.5
50°–60°	1.4	1.7	1.6	1.5	1.5	1.3	1.3	1.4
Activation by DNA (Krishell)	+	+	+	+	+	+	+	+

* The β-glucuronidase activity of each tissue and its extract were, respectively: Ehrlich ascites cell suspension, 3,000 units/ml, 250 units/ml; white Swiss, 3,500 units/gm, 672 units/ml; AKR 650 units/gm, 64 units/ml; C3H, 480 units/gm, 50 units/ml, 129, 3,200 units/gm, 624 units/ml; DBA, 4,000 units/gm, 385 units/ml; stilbestrol-treated AKR 900 units/gm, 280 units/ml, and testosterone propionate-treated A Cloudman 40,000 units/gm, 2,485 units/ml.

† For both 0.1 M acetate and phosphate buffer.

REFERENCES

1. BERNFELD, P., and FISHMAN, W. H. A Coenzyme of Spleen Beta-glucuronidase. Science, **112**:653–54, 1950.
2. ———. Isolation of β-glucuronidase of Calf Spleen. Arch. Biochem., **27**:475–76, 1950.
3. FISHMAN, W. H. Studies on Beta-glucuronidase. II. Factors Controlling the Initial Velocity of Hydrolysis of Some Conjugated Glucuronides. J. Biol. Chem., **131**:225–33, 1939.
4. ———. Beta-glucuronidase and the Action of Steroid Hormones. Ann. N.Y. Acad. Sc., **54**:548–57, 1951.
5. FISHMAN, W. H., and FARMELANT, M. F. Beta-glucuronidase Studies in Inbred Mice. Effects of Androgen and Estrogen. Endocrinology, **52**:536–45, 1953.
6. FISHMAN, W. H.; SPRINGER, B.; and BRUNETTI, R., Application of an Improved Glucuronidase Assay Method to the Study of Human Blood Beta-glucuronidase. J. Biol. Chem., **173**:449–56, 1948.

7. Fishman, W. H., and Talalay, P. A Simplified Method of Preparing Active Extracts of Beta-glucuronidase. Science, **105**:131–32, 1947.
8. Karunairatnam, M. C., and Levvy, G. A. The Inhibition of Beta-glucuronidase by Saccharic Acid and the Role of the Enzyme in Glucuronide Synthesis. Biochem. J., **44**:599–604, 1949.
9. Law, L. W.; Morrow, A. G.; and Greenspan, E. M. Inheritance of Low Liver Glucuronidase Activity in the Mouse. J. Nat. Cancer Inst., **12**:909–16, 1952.
10. Morrow, A. G.; Greenspan, E. M.; and Carroll, D. M. Liver Glucuronidase Activity of Inbred Mouse Strains. J. Nat. Cancer Inst., **10**:657–62, 1949.
11. Riotton, G., and Fishman, W. H. Beta-glucuronidase Studies in Inbred Mice: Androgenic Hormones and Kidney and Urinary Beta-glucuronidase Activity. Endocrinology, **52**:692, 1953.
12. Talalay, P.; Fishman, W. H.; and Huggins, C. Chromogenic Substrates. II. Phenolphthalein Glucuronic Acid as Substrate for the Assay of Glucuronidase Activity. J. Biol. Chem., **166**:757–72, 1946.

6-Mercaptopurine: Effects in Mouse Sarcoma 180 and in Normal Animals*

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The possibility that the control of neoplasia might be achieved with compounds acting as antimetabolites in nucleic acid synthesis has stimulated investigation of the chemotherapeutic activity of unnatural purines and purine analogs. This concept is supported by observations describing the inhibitory actions of 2,6-diaminopurine (5) and 8-azaguanine in rodent tumors (14, 19, 20, 33). Both agents also inhibit the growth of certain micro-organisms, and this effect can be prevented specifically and more or less competitively by adenine (9) or guanine (12, 17, 18, 29), respectively (especially at the higher concentrations of the analogs). Similarily, diaminopurine behaves as an adenine antagonist in such diverse systems as the proliferation of the virus-like "kappa" bodies in *Paramecium aurelia* (32), estrogen-induced growth in chick oviduct (16), and mammalian cells in tissue culture (2). Finally, the destructive action of 2, 6-diaminopurine in mammalian bone marrow (6, 24) and intestinal epithelium (24) may be ascribed to selective injury of cells dependent for growth on high rates of nucleic acid synthesis.

A continuing search for more effective purine antimetabolites has revealed that 6-mercaptopurine (Chart 1) is an inhibitor of the Crocker mouse sarcoma (S-180). 6-Mercaptopurine (10) also inhibits the growth of *Lactobacillus casei*, and this action is prevented more or less competitively by any of the four physiological purine bases. It has been found more inhibitory to a diaminopurine-resistant strain of *L. casei* than to the wild strain (10, 12); studies with a 6-mercaptopurine-resistant strain have indicated that the agent prevents utilization of hypoxanthine primarily and of adenine secondarily, while having little effect on the direct utilization of guanine and xanthine (11). It is an effective inhibitor of embryogenesis in *Rana pipiens* during earliest stages of development (1).

The action against S-180 has proved unique since, hitherto, purine antagonists have proved relatively ineffective in this tumor (30). Moreover, treatment of mice with tolerated doses of 6-mercaptopurine (SK 5357[1]) renders S-180 nonviable; this is indicated by failure of the tumor to "take" when reinoculated into normal mice and by extension of the survival time and production of a significant number of complete recoveries in treated animals. The present report deals with these effects as well as a description of the toxicology of SK 5357 in mice, rats, cats, and dogs.

MATERIALS AND METHOD

Experimental subjects included Swiss-Webster (CFW) mice (Blue Spruce Farms or Millerton Industries), weighing 18–25 gm., Wistar male rats (Carworth Farms), 180–300 gm., and adult, mongrel dogs and cats. Samples of SK 5357, conforming in chemical purity to the synthetic products described by Elion *et al.* (8), were used as the monohydrate and as the anhydrous base; all doses were calculated as the purinemonohydrate. In rodents the purine was usually given as finely dispersed suspensions. These were prepared either by grinding in Potter glass homogenizers with solutions of 0.5 per cent carboxymethyl cellulose[2] in 0.9 per cent NaCl (CMC suspensions) or by dissolving and reprecipitating, in sequence, with equimolar amounts of NaOH and HCl (saline suspensions). Alkaline solutions were employed for intubation by mouth or for intravenous administration. The agent was dissolved with 1.1 M equivalents of NaOH in isotonic NaCl immediately before use.

The schedule of dosage followed in studies of tumor inhibition will be described below. In toxicologic studies various doses were administered in mice and rats in the constant volume of 0.01 ml/gm. LD_{50} values were calculated, according

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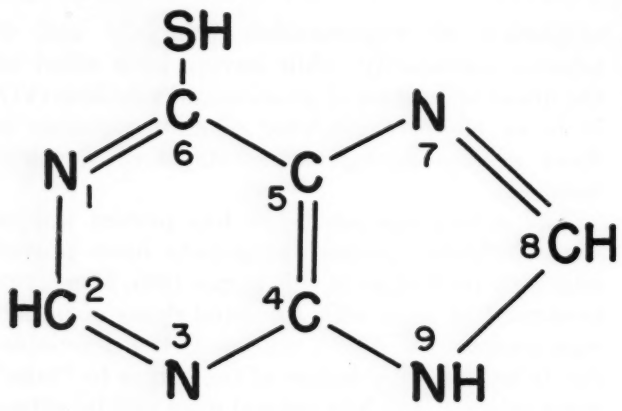
[1] 6-Mercaptopurine will subsequently be referred to as SK 5357.

[2] Cellulose Gum, High Viscosity, Type 120. Hercules Powder Co.

to Litchfield and Wilcoxon (21), after administration of a sufficient number of doses, decreasing in series by a factor of ½, to obtain both 100 and 0 per cent mortality during 14 days of observation. At least ten mice and six rats were employed per dose.

Standard hematologic measurements (38) and microscopic studies were made with blood and tissues from mice and rats anesthetized by ether and sacrificed by exsanguination. Blood was drawn from the abdominal aorta into heparinized syringes. Blood samples from dogs were obtained without stasis from jugular veins of preprandial animals while under sodium thiopental anesthesia. Autopsy specimens of femur and sternum from rats and mice and vertebra and rib from dogs were fixed with Vandergrifft's reagent. Other tissues were fixed in Zenker-formol. All sections were stained with hematoxylin-eosin. In addition to bone marrow the tissues studied microscopically included specimens of S-180, skeletal and



6-MERCAPTOPURINE
(SK 5357)

CHART 1

cardiac muscle, thyroid, thymus, lung, esophagus, testis, lymph nodes, and all abdominal viscera.

Protein, nonprotein nitrogen, and chloride in plasma and glucose in blood were analyzed as in previous work (37). Total plasma bilirubin (i.e., 15-minute reaction in dilutions of plasma treated with methyl alcohol) was determined according to Malloy *et al.* (22). Hepatic function tests, using sodium sulfobromophthalein were performed in dogs as follows: 20 minutes after intravenous administration of 5 mg/kg of the dye, venous blood was drawn without stasis by means of a heparinized syringe; 2 ml. of the sample were mixed with 8 ml. of isotonic NaCl; after centrifugation, two 2-ml. aliquots of supernatant fluid were introduced into each of 2 cuvettes; the first aliquot was diluted with 1 ml. 0.1 N HCl, the second, with 1 ml. 0.1 N NaOH; optical density of the latter was compared to that of the former in the Beckman spectrophotometer (model DU) at 580 mμ; the value obtained was used to calculate the sulfobromophthalein concentration in the original blood sample.

Tumor inhibition test.—Inhibition of S-180 by 6-mercaptopurine was first observed in a screening program which has been described elsewhere (30). The present work represents an extension of these observations in which treatment with the agent was initiated in groups of female mice (CFW, 18–22 gm.) 24 hours after subcutaneous implantation of tumor and was continued for 7 consecutive days. Controls, bearing implants of the same tumors, were treated simultaneously with the corresponding diluent. Various doses in the constant volume of 0.5 ml/20 gm mouse were given once daily by intraperitoneal injection. One day after the final dose vernier calipers were employed to measure (to the nearest 0.1 mm.) the length of the two largest perpendicular axes of each tumor, and from these values an average diameter was calculated.

RESULTS

Effects of 6-Mercaptopurine in Sarcoma 180

Inhibition of growth.—Table 1 summarizes the effects of SK 5357, in various doses, on the growth of S-180 as employed in the inhibition test described above. The doses induced significant and reproducible reductions in tumor size. Moreover, the effects of the higher doses were not remarkably greater than those obtained with 25 or 50 mg/kg/day. Doses greater than 50 mg/kg/day produced intoxication in treated animals which was manifested by enhanced weight loss and mortality during the period of observation. Furthermore, the table demonstrates that CMC and saline suspension of SK 5357 were similar in producing inhibition of growth of S-180 and weight loss and mortality.

TABLE 1

TUMOR INHIBITION AND TOXICITY OF 6-MERCAPTOPURINE IN SARCOMA 180-BEARING MICE

(Treatment initialed 24 hours after implantation and continued for 7 consecutive days)

Dose (mg/kg/day)	Tumor diameter at end of therapy (mm.±S.D.)	Average weight change at end of therapy (gm.)	Mortality during therapy	Cumulative mortality thru 2d week
I. CMC suspension:				
100	4.1±1.4	−2.5	10/30	25/30
Controls	10.8±1.2	±0.0	0/30	7/30
75	5.8±1.7	−1.5	3/30	24/30
Controls	11.5±1.1	−0.5	1/30	11/30
50	7.1±2.2	−0.5	0/30	0/30
Controls	10.5±1.9	+2.0	0/30	8/30
II. Saline suspension:				
125	6.8±1.6	−3.0	6/50	44/50
Controls	13.0±0.5	−1.0	0/5	0/5
100	7.0±1.9	−3.0	14/50	49/50
Controls	12.3±1.6	+1.0	0/5	0/5
75	7.4±1.7	−2.0	3/50	26/50
Controls	13.0±2.1	±0.0	0/5	2/5
50	7.8±1.6	−1.0	1/50	7/50
Controls	12.4±1.5	±0.0	0/5	1/5
25	8.4±1.5	+0.5	0/50	4/50
Controls	11.0±1.0	+1.0	0/5	1/5

Inhibition of growth of S-180 induced by SK 5357 during the 7 days of treatment was not so

marked as has been described for certain other agents, e.g., triethylene melamine, N,N′,N″-triethylene phosphoramide, and 4-aminofolic acids (3, 4, 31). Unlike the latter inhibitors, however, SK 5357 induced an effect in the tumor which was persistent and which frequently resulted in a loss of viability of tumor cells.

Table 2, presented in two parts, illustrates the therapeutic effects of 50 mg/kg/day of SK 5357. Part I shows the moderate inhibition of growth during the treatment period initiated 24 hours following implantation of S-180. Of greater interest is the distribution of deaths caused by S-180: SK 5357 notably retarded the lethal processes of S-180. Although a majority of the control mice were dead by the end of 3 weeks, it was not until the end of 6 weeks that an equal number of treated mice had expired.

Part I of Table 2 also indicates that the recovery[3] rate of treated animals, 30 per cent, was greater than that of controls, 13 per cent. Recovery in the controls was characterized by ulceration of the skin and extrusion of the underlying tumor mass. However, recovery in the treated group began as a resorptive process in tumors which had previously been growing for 2–4 weeks; resorption was completed within approximately 1 week. Enhanced recovery as the result of tumor resorption has been observed consistently in groups of mice treated with nonlethal doses of SK 5357 (see statistical summary below).

The second part of Table 2 depicts the effects of therapy with SK 5357 instituted 96 hours after implantation of S-180. (At this time S-180 is well established and growing at a maximal rate.[4]) No inhibition of growth was evident at the end of 7 days of treatment. The increased mortality among the treated animals during the second week of the experiment may have been due to combined toxic effects from tumor and agent. The majority of the individuals surviving beyond the second week recovered. Under the conditions of this experiment, the recovery rate was increased from 10 per cent (controls) to 40 per cent (treated animals).

TABLE 2

RECOVERY FROM SARCOMA 180 AND INCIDENCE OF DEATHS OF MICE TREATED WITH SK 5357*

	No. of mice	Tumor diameter: Start of therapy (mm.±S.D.)	Tumor diameter: End of therapy (mm.±S.D.)	Occurrence of deaths by weeks after therapy start						Recovery from tumor†
				2d	3d	4th	5th	6th	7th	
I. Therapy started 24 hrs. after tumor implantation:										
SK 5357	30	trocar piece	7.1±2.2	0	3	3	9	4	2	9
Control	30	trocar piece	10.5±1.9	8	10	7	1	0	0	4
II. Therapy started 96 hrs. after tumor implantation:										
SK 5357	20	5.8±0.7	12.0±1.2	8	0	1	1	2	0	8
Control	20	6.5±0.9	12.8±1.0	4	7	3	1	2	0	2

* CMC suspension. Dose: 50 mg/kg/day × 7 in 0.5 ml. injected I.P.
† Followed for at least 14 days afer tumors were no longer palpable.

In preliminary observations it was found that SK 5357 inhibited S-180 when administered by oral intubation in doses approximately twice those active by intraperitoneal injection. To investigate the possibility that a continuous, low-level intake of SK 5357 might enhance the antitumor activity of the agent, feeding experiments were initiated. Homogeneous mixtures of SK 5357 and ground food (Purina Laboratory Chow) were prepared. Mice were placed on these diets 24 hours after implantation with S-180 and observed daily for manifestations of toxicity; the tumor status was evaluated every 7 days. Control mice were maintained on unsupplemented ground food. Concentrations of SK 5357 equal to or greater than 250 mg/kg of diet proved lethal within less than 14 days.

Chart 2 summarizes results of an experiment wherein mice were fed a diet containing 62.5 mg/kg of SK 5357 for the first 21 days, were placed on an unsupplemented diet between 22 and 27 days, and were returned to the SK 5357 diet for the remainder of the experiment. Inhibition of the growth of tumors in the treated group was only moderate. At the end of the second week of

[3] The authors prefer to employ "recovery" in describing the fate of surviving animals rather than the more absolute term, "cure." "Recovery" seemed equally applicable to the fate of both control and treated animals, whereas "cure" could only have been used to refer to survivors among treated groups. Furthermore, all surviving animals were held for a minimum of 2 weeks to a maximum of 7 weeks after their tumors were no longer palpable. In no instance did tumors recur as judged by the criterion of palpability: nor were tumor masses evident in gross dissections of survivors when experiments were terminated. Since, admittedly, an indeterminate number of such animals may not have been "cured" in the definitive sense, it was deemed more accurate to designate survival, within the framework of the present experimental conditions, as "recovery" from S-180.

[4] Unpublished observations.

the experiment the diameters of the treated tumors were 7.5–17.7 mm.; the range of the diameters of control tumors was 10.8–17.6 mm. However, the survival time of treated animals was longer

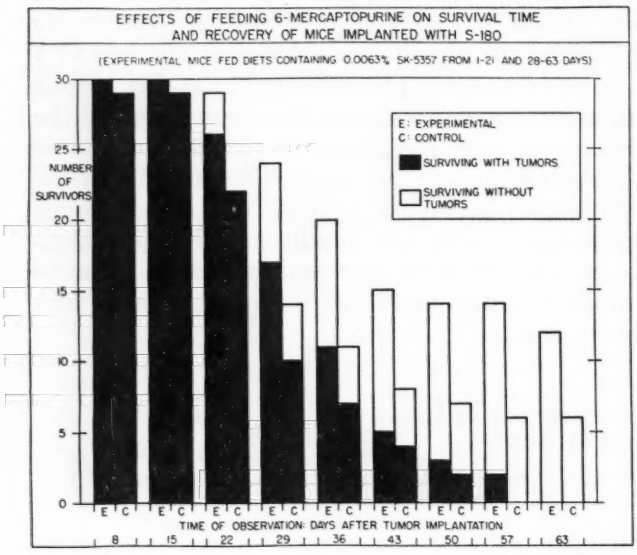

EFFECTS OF FEEDING 6-MERCAPTOPURINE ON SURVIVAL TIME AND RECOVERY OF MICE IMPLANTED WITH S-180
(EXPERIMENTAL MICE FED DIETS CONTAINING 0.0063% SK-5357 FROM 1-21 AND 28-63 DAYS)
E: EXPERIMENTAL
C: CONTROL
SURVIVING WITH TUMORS
SURVIVING WITHOUT TUMORS
NUMBER OF SURVIVORS
TIME OF OBSERVATION: DAYS AFTER TUMOR IMPLANTATION

CHART 2

mice recovered; in each case the tumor masses were extruded as described above. Of the 113 survivors among the 325 treated mice, only seven recovered by the mechanism of extrusion; these seven were distributed as follows: 3, 1, 1, and 2, respectively, in experiments 1, 2, 3, and 7 of Table 3. The remaining 106 survivors among treated animals recovered as the result of tumor resorption. The data in Table 3 provide significant evidence for the conclusion that SK 5357 increased the recovery rate among treated animals. That such an increase took place in *all* of the seven experiments has a 1:128 probability of random occurrence. Furthermore, the mean of the differences in recovery rates is significant with $P < 0.01$ (calculated by the t test [13]).

Histology of treated S-180.—Pronounced cytoplasmic and nuclear changes were noted in the cells of S-180 following *in vivo* exposure to the action of SK 5357. To examine the onset and progression of these changes, tumors were removed at 24-hour intervals following initiation of therapy and were prepared for histological study. SK 5357

TABLE 3

RECOVERY RATE OF MICE TREATED WITH SK 5357

Exp. no.	Experimental conditions	Treated No.	Treated Recovery rate (T) (per cent)	Control No.	Control Recovery rate (C) (per cent)	Difference in recovery rate (T–C) (per cent)
1	25 mg/kg/day×7 by the intraperitoneal route; therapy started 24 hrs. after tumor implantation	80	31.2	50	18.0	+13.2
2	50 mg/kg/day×7, otherwise the same as No. 1	60	28.3	45	8.9	+19.4
3	100 mg/kg/day×7, otherwise the same as No. 1	75	37.3	45	15.5	+21.8
4	50 mg/kg/day×7 by the intraperitoneal route, therapy started 96 hrs. after tumor implantation	30	40.0	25	12.0	+28.0
5	Diet, supplemented with 62.5 mg/kg, fed ad libitum from 24 hrs. after tumor implantation, day 1, through day 21 and day 28 through day 62	30	40.0	30	20.0	+20.0
6	Diet, supplemented with 125 mg/kg, fed ad libitum from 24 hrs. after tumor implantation, day 1, through day 7 and day 15 through day 21	30	40.0	10	20.0	+20.0
7	Diet, supplemented with 250 mg/kg, otherwise the same as No. 6	20	35.0	10	20.0	+15.0
	Totals	325		215		
	Means ± standard error		36.0±1.7		16.3±1.7	+19.6±1.8

than that of the controls, and the number of recoveries was doubled. As in experiments described above, recovery in treated animals occurred by resorption of the tumor—in controls, by extrusion of hemorrhagic, necrotic, tumor masses.

Table 3 summarizes the recovery rates obtained in all experiments in which treated mice were held sufficiently long to evaluate the effect of treatment on survival. Among the 215 controls thirty-three

was injected intraperitoneally in a dose of 100 mg/kg/day contained in 0.5 ml. of CMC suspension; treatment was started 24 hours after tumor implantation.

Changes evident in tumors treated for 24 or 48 hours were essentially the same. Moderate stromal edema and slight enlargement of both nucleus and cytoplasm had occurred. After 72 hours the stromal edema was more marked and cellular en-

largement more evident. Major changes were found at 96 hours; nuclear aberrations, such as hyperchromasia and pyknosis, were prominent, and giant-cell formation was seen. The edema previously noted was less marked.

At 120 hours the tumors were largely composed of giant cells, many of which were multinucleated. Nuclei were large and possessed varied and bizarre chromatin patterns; there was little or no edema. Tumors at 144 hours were essentially similar in appearance; cell boundaries were more sharply defined, and nuclear and cytoplasmic vacuoles had appeared in some cells. A few of the giant cells had irregular, stellate cytoplasmic processes and bizarre, deeply basophilic, peripherally located nuclei. By 192 hours this type of cell predominated in treated tumors (compare Figs. 1 and 2).

Sarcoma 180, even during early stages of growth in the mouse, contains small areas of necrosis which increase in size and incidence as the tumor develops. These necrotic areas are particularly evident at 168–192 hours after implantation.[4] Of interest is the fact that the treated tumors described above were free of necrosis.

Resistance of S-180 to SK 5357.—The evidence marshalled above demonstrates that SK 5357 inhibits the growth of S-180 to a significant, though moderate, extent; causes marked histological alteration in the majority of tumor cells; prolongs survival time of the host; and induces complete resorption of one-third of the treated tumors. What factors accounted for therapeutic failures in the remaining two-thirds? Certain of these failures may be attributed to toxicity of SK 5357 *per se* or to a combination of its effects and the morbidity produced by S-180 itself. On the other hand, there is evidence for a rapid appearance of resistance to SK 5357 in treated tumors.

The first indication of resistance was obtained in experiments designed to test the "viability" of treated tumors after reinoculation into normal mice. Implants from untreated tumors almost invariably "take" and grow in normal, CFW mice (35). However, following therapy with SK 5357 (100 mg/kg/day for 7 consecutive days) this capacity was largely impaired. A series of 33 mice surviving therapy in various experiments has served to illustrate this point. On the day following the last injection, tumors were removed, and those presenting the most favorable gross appearance were used for transplantation into normal mice. Thus trocar pieces from 21 tumors were implanted into 33 normal mice. In 30 of these mice tumors failed to appear. In the remaining three mice, slowly growing tumors appeared which did not exceed the size of the original implant until the 3d week. The delayed appearance and reduced rate of growth raised the question whether these tumors might have been altered strains of S-180, possibly resistant to SK 5357, or whether the implants at time of transplantation might have contained inhibitory amounts of mercaptopurine or of an active derivative arising from its metabolism. No conclusive evidence has been obtained in favor of either possibility. However, a demonstration of the origin of resistance in S-180 has been made and is described below.

For this demonstration a tumor which had grown in a bioassay experiment similar to the one just described was selected for further study. As shown in bioassay 1, Chart 3, this tumor was allowed to grow for 28 days after implantation without further treatment. At this time the tumor which had grown to diameters of 9.6 mm. × 10.1 mm. was removed and transplanted into five normal mice, bioassay 2. All implants grew slowly; after 8 days of growth the mean of the averaged diameters was 7.4 mm. (S.D., ±0.4) as compared to a similar parameter for five normal control tumors of 12.0 mm. (S.D., ±1.7). Low-dose therapy with SK 5357 (25 mg/kg/day) was initiated at this time and continued as shown in bioassay 2, Chart 3. In subsequent bioassays, although *all* implants grew, it was customary to select for passage only that tumor of each group which by gross appearance was judged most satisfactory.

The 25 mg/kg/day dose was employed in bioassay 3. In subsequent bioassays doses were increased to 100—125 mg/kg/day. In spite of the increased dosage *all* transplants grew in bioassays 5 through 7. These results were in contrast to the impaired capacity for growth described above in the case of transplants derived from S-180 which had received but one course of treatment. Such a difference of response to the test of transplantation was, by itself, persuasive evidence for the development of resistance. Additional evidence was obtained from the observation that in spite of treatment with large doses, transplants grew in bioassays 5 through 7 at rates comparable to those of the untreated implants designated as "controls" in Chart 3. For example, at the end of therapy in bioassay 6 the average diameter of treated tumors was 7.8 mm. (S.D., ±0.5); of untreated "controls," 8.3 mm. (S.D., ±1.2). The insignificant inhibition produced by SK 5357 in this experiment was in contrast to its effects in normal S-180 (Tables 1 and 2).

Each of the treated tumors from bioassay 6 served as a mouse-to-mouse donor for the experi-

mental group in bioassay 7. In addition, five transplants obtained from the tumor of mouse 3 were employed as untreated "controls" (Chart 3). At the end of treatment in bioassay 7 the average

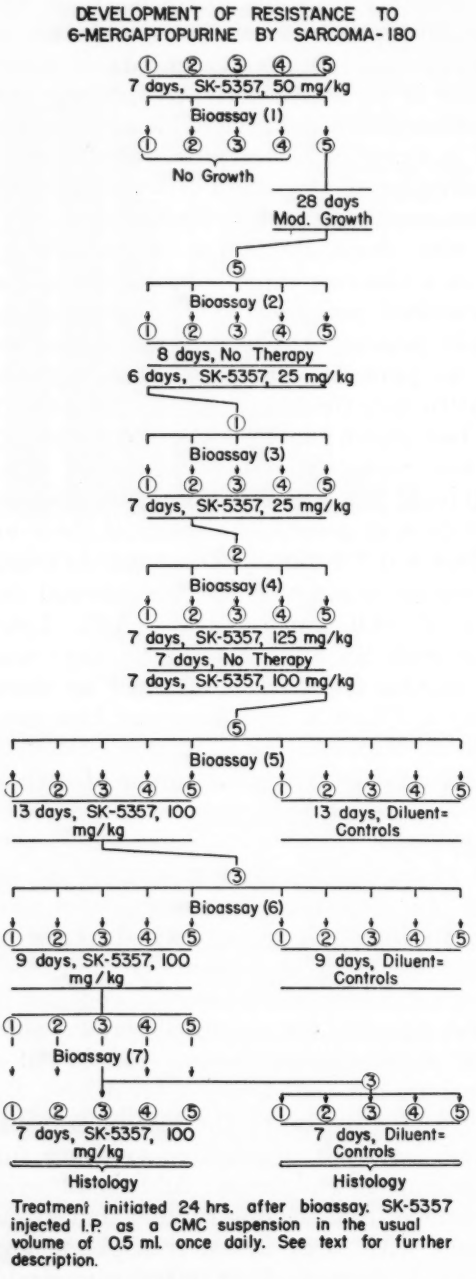

DEVELOPMENT OF RESISTANCE TO
6-MERCAPTOPURINE BY SARCOMA-180
7 days, SK-5357, 50 mg/kg
Bioassay (1)
No Growth
28 days
Mod. Growth
Bioassay (2)
8 days, No Therapy
6 days, SK-5357, 25 mg/kg
Bioassay (3)
7 days, SK-5357, 25 mg/kg
Bioassay (4)
7 days, SK-5357, 125 mg/kg
7 days, No Therapy
7 days, SK-5357, 100 mg/kg
Bioassay (5)
13 days, SK-5357, 100 mg/kg
13 days, Diluent= Controls
Bioassay (6)
9 days, SK-5357, 100 mg/kg
9 days, Diluent= Controls
Bioassay (7)
7 days, SK-5357, 100 mg/kg
7 days, Diluent= Controls
Histology
Histology
Treatment initiated 24 hrs. after bioassay. SK-5357 injected I.P. as a CMC suspension in the usual volume of 0.5 ml. once daily. See text for further description.

CHART 3.—Transplantation and treatment history of a tumor with apparent resistance to SK 5357.

diameters of experimental and "control" tumors were 6.9 mm. (S.D., ±0.6) and 7.4 mm. (S.D, ±0.5), respectively.

All tumors obtained in bioassay 7 were removed for histological study; Figures 3 and 4 illustrate, respectively, the microscopic appearance of the treated and "control" groups. The "controls" (Fig. 4) were similar to normal S-180 (Fig. 1); the treated, *resistant* tumors differed from normal S-180 merely in respect to a moderate increase in cytoplasmic vacuolization (Fig. 3). This moderate change was in contrast to the marked alteration induced in normal S-180 by SK 5357 (Fig. 2).

Antagonism and/or synergism.—Efforts either to oppose or to enhance the activity of SK 5357 against S-180 have, to date, proved unsatisfactory. Various compounds were given just prior to or simultaneously with SK 5357 (100 mg/kg/day, intraperitoneally, for 7 days). These included the following (mg. values after each compound indicate daily dose/kg): adenine (100 mg.), guanine (250 mg.), xanthine (500 mg.), hypoxanthine (250 mg.), 2,6-diaminopurine (35 mg.), 8-azaguanine (50 mg.), citrovorum factor (5 mg.), and folic acid (25 mg.). Treatment of mice with SK 5357, 50 mg/kg/day for 4 days prior to implantation of tumor, had no effect on the growth of S-180. Nor did a single large dose (125 mg/kg) 24 or 96 hours after tumor implantation, followed by nine daily doses of 50 mg/kg, significantly alter the tumor inhibition to be seen with the lower dose alone.

Toxicology of 6-Mercaptopurine in Mice Implanted with Sarcoma 180

The data of Table 1 indicated that doses of 6-mercaptopurine greater than 50 mg/kg/day caused weight loss and shortened the survival time of mice with tumors. In order to study the pathologic changes, mice were given, beginning 24 hours after implantation of tumor, seven successive daily doses of 50, 100, or 125 mg/kg/day and were sacrificed 24 hours after the final dose. Granulocytopenia and reticulocytopenia were the outstanding alterations of the blood cells (Table 4), and microscopic observations of sternal bone marrow revealed hypoplasia (20–70 per cent depletion in cellularity). Involution was found in the thymus, but no significant histologic changes were evident in lymph nodes or spleen. A slight swelling of the nuclei appeared in glandular epithelium of small and large intestine of individuals given doses of 100 or 125 mg/kg/day.

Toxicology of 6-Mercaptopurine in Normal Mammals

Mice.—The data summarized in Table 5 indicate that saline and CMC suspensions of SK 5357 were not significantly different in toxicity. The agent, when given by oral intubation, proved somewhat less toxic than by intraperitoneal administration. Limited studies with the AKR strain suggested that both sexes were equally susceptible to intoxication and that AKR mice were some-

what more resistant to SK 5357 than the CFW mice.

The course of fatal intoxication following single doses of SK 5357 proved similar regardless of the strain of mice or the route of administration employed. Of 150 CFW and AKR animals, given 250 or 500 mg/kg either intraperitoneally or by oral intubation, the majority, 87, succumbed between 4 and 7 days after intoxication. Only eight fatalities were recorded earlier than 72 hours, and fourteen others were noted during the 2d week. Fatal doses did not elicit significant changes during the first 6 hours. However, at 24–48 hours after 500 mg/kg were administered intraperitoneally, all mice were weak and unkempt in appearance, and a significant loss in body weight was observed. These manifestations progressed in severity, and terminally animals were found to have decreased in weight by 30–40 per cent. Diarrhea was also noted during the final day of survival. In individuals surviving after single doses of 125 or 250 mg/kg, transient losses in weight were observed during the 1st week which were subsequently recovered in the 2d week after injection.

To compare pathologic alterations caused by 6-mercaptopurine in mice with those elicited in rats (see below), a small number of CFW males were given 500 mg/kg by oral intubation and were sacrificed in groups of 5 at 24, 48, and 72 hours. No macroscopic tissue changes were found at the time of sacrifice. Cell counts in the blood at 72 hours revealed marked reticulocytopenia and moderate elevation of the hematocrit. Microscopic study of the animals killed at 72 hours revealed the following: moderate hypoplasia of sternal bone marrow (30–40 per cent depletion in cellularity), thymic involution, and, in the small and large intestine, swelling of nuclei in surface and glandular epithelium as well as edema, congestion, and leukocytic infiltration of the mucosa. A small number of intestinal glands in each animal were dilated and contained necrotic debris. The changes described above were less marked in the large than in the small intestine.

TABLE 4

BLOOD CELLS IN S-180-BEARING MICE TREATED WITH 6-MERCAPTOPURINE*

(Mean Values ± Standard Deviations)

GROUP	RBC (cells/c mm × 10^{-6})	RETICULOCYTES (per cent)	WBC (cells/c mm × 10^{-3})	DIFFERENTIAL Monos (per cent)	DIFFERENTIAL Polys (per cent)
Control	10.0±1.5	2.8±1.8	4.5±1.1	68	32±6.4
SK 5357	8.6±1.5	0	1.1±0.7	98	2±2.6

* Ten mice in each group. Treatment initiated 24 hours after tumor implantation. Saline suspension, 100 mg/kg/day, injected I.P. in 0.5 ml. for 7 days. Blood from abdominal aorta.

TABLE 5

TOXICITY OF 6-MERCAPTOPURINE IN MICE AND RATS

Species	Route of administration	Preparation	No. of successive daily doses	LD_{50} (mg/kg/day)	19/20 Confidence limits* (mg/kg/day)	S*
Mice, CFW male	intraperitoneal	saline suspension	1	227	199–259	1.24
	intraperitoneal	saline suspension	5	98	82–117	1.21
	intraperitoneal	CMC suspension	1	275	224–338	1.27
	oral	alkaline solution	1	338	†	1.21
Mice, AKR male	intraperitoneal	CMC suspension	1	350	†	1.10
Mice, AKR female	intraperitoneal	CMC suspension	1	350	†	1.10
Rats, Wistar male	intraperitoneal	saline suspension	1	255	199–327	1.25
	intraperitoneal	saline suspension	5	59	44– 78	1.28
	oral	alkaline solution	1	382	268–546	1.37

* Calculated according to Litchfield and Wilcoxon (21).

† Confidence limits not calculated since expected mortality for doses used was either >84 per cent or <16 per cent (21).

Rats.—The results presented in Table 5 indicate that rats and mice are equally susceptible to 6-mercaptopurine. However, the course of fatal intoxication in the former species was shorter in duration than that in mice. For example, the majority of fatalities among rats given lethal doses occurred between 24 and 48 hours after intraperitoneal administration and between 72 and 96 hours after oral intubation (250–555 mg/kg). Like mice, fatally intoxicated rats exhibited progressing weakness and loss of body weight. In addition, rats became dyspneic within 24 hours after injection and continued, until time of death, to give evidence of severe respiratory embarrassment—a manifestation of intoxication not found in mice or in cats and dogs (see below).

Pathologic alterations caused by SK 5357 were studied in the following groups of rats: (A) twelve given two successive daily doses of 100 mg/kg/day intraperitoneally and sacrificed at 48 hours; (B) six given the same dose but sacrificed at 96 hours; and (C) six intubated orally with 500 mg/kg and sacrificed at 72 hours. Severe dyspnea was evident in each animal before sacrifice and was associated with variable amounts of pleural effusions. For example, it was possible to withdraw by syringe between 2 and 7 ml. of clear fluid from the thorax of each group C rat. The fluid formed soft clots and contained 100–108 meq/1 Cl^- and 2.9–3.6 gm protein/100 ml. Gross lesions were noted in the lungs of only six of the 24 animals sacrificed; these changes included scattered gray-yellow areas of consolidation in five, edema in one, and atelectasis in one. Edema of the thymus was also prominent in animals of groups B and C. Bone marrow expelled from their femora was dark red and fluid. Liver lobes of group C animals were rounded and exhibited numerous superficial areas of reddish-yellow discoloration. Finally, the rats of groups A and B, which had received intraperitoneal injections, contained accumulations of peritoneal fluid and adhesions among abdominal organs. This evidence of peritonitis, confirmed subsequently by microscopic observations, probably resulted from a direct inflammatory action of 6-mercaptopurine, since it was not found in animals given the agent by oral intubation (group C). It is interesting to note that direct damage to the peritoneal lining has been previously reported to occur in rats given adenine by intraperitoneal injection (26).

TABLE 6

HEMATOLOGICAL FINDINGS IN RATS GIVEN 6-MERCAPTOPURINE

(Mean Values ± Standard Deviations)

Group	Reticulocytes (per cent)	Hematocrit (ml/100 ml)	Neutrophils (cells/c mm×10^{-3})	Lymphocytes (cells/c mm×10^{-3})
Control*	2.1±0.4	45±2.4	0.9±0.3	4.9±1.6
A†	1.8±1.1	57±5.6	1.2±0.5	2.2±1.3
B†	0	62±3.5	1.0±0.6	0.4±0.1
C†	0.3±0.2	64±9.2	1.9±0.6	1.4±0.8

* Thirteen animals.
† See text for treatments.

Hematologic alterations, as seen in Table 6, included reticulocytopenia, lymphopenia, and elevation of the hematocrit. In view of the coincident pleural effusions the last mentioned change was not unexpected. Although the neutrophil count was not depressed, the granulocytes were observed to be uniformly hypersegmented. Sternal bone marrow of the three groups of rats appeared hypoplastic in histologic sections (20–75 per cent depletion in cellularity), and involution of the thymus was prominent. Microscopic changes were also observed in the small and large intestines of all rats; these were most marked in group B, in which they resembled lesions described above in mice given 500 mg/kg by oral intubation.

Additional histologic changes were evident in liver, lung, and heart; these lesions—to be described in detail elsewhere[5]—will be summarized briefly herein. In *liver*, areas of diffuse necrosis occurred principally, but not exclusively, in subcapsular regions. The lesions involved numerous adjacent lobules in each of which damage was diffuse in distribution. Necrotic areas were surrounded by polymorphonuclear leukocytic infiltrations. Hepatic damage was found in seven of the twelve rats of group A and in all animals of groups B and C; it was most extensive in Group C. In *lungs*, edema was observed in perivascular and peribronchial tissues, and was also present in perithymic tissues. Fluid in alveoli occurred in only one rat in Group C.[6] Such microscopic alterations were not found in mice and dogs. In *heart*, acute myocarditis was seen in five of the six rats in group C.

Cats.—Acute manifestations were not observed in two pairs of cats given intravenous injections of 50 and 100 mg/kg of SK 5357, respectively. During the following 7 days the animals survived, though the pair given the higher dose became anorexic, lost weight, and appeared "unhealthy." Observations were terminated at the end of this period.

Dogs.—Intoxication was studied in ten dogs receiving repeated intravenous injections of SK 5357. In two animals given three successive daily doses of 50 mg/kg/day, emesis was the earliest disturbance noted. Episodes of vomiting began within 5 hours after the first dose and continued intermittently thereafter. Both animals failed to eat their usual daily allotment of food and lost weight progressively. During the 4th day following the initiation of treatment, each animal manifested bloody diarrhea. One individual succumbed by the end of this day; the other, listless, weak, and obviously ill, was sacrificed for pathologic study. Marked hemoconcentration was found in the latter dog's blood obtained immediately before sacrifice; the hematocrit had increased to 65 per cent from a pretreatment value of 45 per cent. Its

[5] S. S. Sternberg, D. A. Clarke, and F. S. Philips, to be published.

[6] Additional studies of the pathogenesis of pulmonary damage in rats have revealed necrotic changes in alveolar septa.

plasma was bright yellow and contained an excessive concentration of bilirubin (8.8 mg/100 ml). The principal findings at autopsy included yellow discoloration of the renal medulla, hemorrhages in mesenteric lymph nodes, dark, bloody fluid in small and large intestine, and numerous hemorrhages in the mucosa of the colon. Microscopic changes will be described below. A second group of dogs was given four successive daily doses of 25 mg/kg/day, and the pertinent findings obtained are listed in Table 7. Although anorexia in all and emesis in dogs 1 and 2 were noted during the first week of intoxication, none of the animals developed hemorrhagic diarrhea. Nevertheless, an inspection of Table 7 reveals certain other morbid changes shared by each dog. Among these were weight loss, hemoconcentration, granulocytopenia (except in dog 2), hypochloremia, hyperbilirubinemia, and increased retention of sodium sulfobromophthalein. During the period when both sulfobromophthalein retention and plasma bilirubin were elevated, each dog exhibited choluria and a yellowish hue in sclera and buccal mucosa. As an example, bilirubin in a concentration of 5 mg/100 ml was found in urine of dog 3 collected during the 8th day.

By the end of the 2-week periods included in the results of Table 7, dogs 1, 3, and 4 evidenced recovery from serious manifestations of intoxication. They were observed for an additional 2 weeks during which they continued to improve. The most seriously intoxicated individual, dog 2, was sacrificed at 7 days. Its autopsy revealed yellow discoloration of organs such as the liver, kidney, and aorta; dark, bloody fluid in small intestine; and tarry, inspissated stool in colon. No other gross changes were noted.

A final group of four dogs was given nine doses of 10 mg/kg/day during a period of 10 days. These animals survived and exhibited only moderate weight loss and no significant alterations in plasma chloride, plasma bilirubin, or sulfobromophthalein retention. Two weeks after initiation of treatment granulocyte counts were moderately decreased to levels between 38 and 58 per cent of initial values, and lymphopenia was present in two of the four dogs. At this time hematocrit values were found to have fallen by 15, 20, 24, and 27 per cent, respectively. In two of the animals, hematocrits were determined again 10 days later without evidence of further change. The rapid appearance of anemia in these individuals and in dog 1 (Table 7) suggested that suppression of erythrogenesis was not the sole factor involved. It is conceivable that intestinal bleeding may have contributed to red cell losses.[7]

TABLE 7

EFFECTS OF 6-MERCAPTOPURINE GIVEN INTRAVENOUSLY IN DOGS

(25 mg/kg/day at 0, 1, 2, and 3 days)

Dog	Day of observation	Weight (kg.)	Blood			Plasma		Sulfobromophthalein test*
			Hematocrit (ml/100 ml)	Neutrophils (/mm³×10⁻³)	Lymphocytes (/mm³×10⁻³)	Chloride (meq/l)	Bilirubin (mg/100 ml)	(mg/100 ml)
1	0	16.1	41	8.4	3.8	118	0.15	0.26
	4	15.0	45	6.3	2.1	104	1.75	1.32
	7	14.8	40	0.5	3.9	102	1.70	1.81
	11	15.0	27	0.2	5.1	118	0.50	0.73
	14	15.5	24	8.7	6.9	120	0.35	0.29
2	0	15.0	46	4.8	2.1	116	0.15	0.38
	4	14.1	59	7.6	0.1	101	1.00	1.35
	7 S	13.4	72	5.0	0.6	94	8.20	2.72
3	0	15.2	50	5.5	4.4	118	0	0.28
	4	14.4	54	4.1	0.5	105	0.90	1.88
	7	13.6	60	1.1	0.6	100	4.00	
	10	13.2	54	7.4	3.8	97	4.10	2.55
	15	12.7	47	4.3	3.0	106	0.60	0.74
4	0	14.1	51	11.8	0.5	126	0.50	0.25
	4	13.4	57	10.1	0.2	100	1.95	2.54
	7	12.6	57	3.0	1.1	102	2.05	2.75
	10	12.5	49	8.2	0.7	89	2.10	2.68
	15	12.3	45	12.5	2.4	104	0.65	1.84

S = sacrificed.

* A concentration of 1 mg/100 ml of blood may be equated to an approximate "retention" of 20 per cent.

By the end of the 2-week periods included in the results of Table 7, dogs 1, 3, and 4 evidenced recovery from serious manifestations of intoxication. They were observed for an additional 2 weeks during which they continued to improve. The most seriously intoxicated individual, dog 2, was sacri-

[7] The possibility was considered that SK 5357 might be a hemolytic agent. However, gross hemolysis was not found in blood samples obtained routinely from various mice, rats, and dogs used in the current study. Furthermore, in the dogs given successive doses of 10 mg/kg/day, fragility of erythrocytes to hypotonic solutions of NaCl (38) remained unaltered during and after treatment. Nor did erythrocyte fragility change in rats given 100 mg/kg intravenously and sacrificed in pairs at ½, 2, and 24 hours, respectively.

In the two above animals, sacrificed for histologic study, intestinal lesions were the principal findings. In the small intestine these included widespread denudation of surface epithelium, congested and dilated capillaries in tips of denuded villi, swelling and atypia of glandular nuclei, leukocytic infiltration throughout the mucosa, and, in dog 2 (Table 7), regenerative changes in surface epithelium. The colons of both dogs showed only moderate glandular atypia, intact surface epithelium, and, in the dog given 50 mg/kg/day, numerous recent hemorrhages in the mucosa. Other histologic alterations included moderate depletion of cellularity in vertebral bone marrow[8] and occasional, small areas of focal hepatic necrosis in dog 2.

DISCUSSION

Sarcoma 180 has in the past proved unusually resistant to chemotherapeutic measures. Although a number of agents have been described as inhibitors of S-180, none has been shown to damage this tumor to such an extent that there occur both complete disappearance of the neoplasm and recovery of treated animals. Nor have such agents been as effective against older, well vascularized and growing tumors as they are against 1-day-old transplants. Against this background the evidence adduced above reveals that 6-mercaptopurine is a novel substance. However, its "curative" effects against S-180 are not unique among chemotherapeutic actions of different agents against other transplantable rodent tumors. For example, 4-aminopteroylglutamic acid has been shown to cause complete destruction of the rat sarcoma R39 (34). Certain compounds with nitrogen mustard activity have caused similar results in the same tumor as well as in other rat sarcomas and carcinomas (35). 2,4,6-Triethylenimino-*s*-triazine, a compound with nitrogen mustard-like activity, has caused significant destruction of carcinoma 1025 in the mouse and, in rats, has caused complete recoveries from sarcoma R39, Jensen sarcoma, Flexner-Jobling carcinoma, and the Walker carcinosarcoma 256 (36), as well as the sarcoma 231 (7).

The actions of 6-mercaptopurine in S-180 may be related to and possibly understood by its effects in other biological systems. Studies of growth inhibition with *Lactobacillus casei* suggest that the agent may act as an antimetabolite in purine metabolism (10). As mentioned above, the compound appears to prevent utilization of hypoxanthine, primarily, and of adenine, secondarily, while having little or no effect on the metabolism of guanine and xanthine (11). Its locus of action can be distinguished from that of another adenine analog, 2,6-diaminopurine (9), by the lack of cross-resistance shown in strains of *L. casei* made resistant to each of the drugs (11, 12).

Moreover, 6-mercaptopurine is an inhibitor of xanthine oxidase but is oxidized by this enzyme to (apparently) 6-mercaptouric acid.[9] There are, thus, a number of independent points in purine metabolism at which the compound might act and at which the inhibition of the neoplastic cell might occur. The possibility also exists that its mechanism of action may be related to the mercapto group rather than to the purine moiety, i.e., become involved in sulfhydryl metabolism in some as yet unknown way. The *bis*-purinyl-disulfide (formed by oxidation of 6-mercaptopurine) has tumor-inhibiting properties similar to those of the mercaptan; this fact, however, casts little light on the relationship of these substances to sulfhydryl metabolism, since the disulfide undergoes spontaneous dismutation to regenerate 6-mercaptopurine.

The clear-cut antagonism between 6-mercaptopurine and natural purines in the growth of *L. casei* suggested several reversal experiments with natural purines, folic acid, and leucovorin, and combined therapy with other purine antagonists in trials with S-180. The negative outcome of these experiments neither supports nor disproves the hypothesis that the mode of action of the compound may be similar in the two biological systems, but may result from failure to supply the specific metabolite affected by the drug.

The demonstration that—as with *L. casei*—mercaptopurine-resistant strains of S-180 can be obtained with facility may help explain the failure of the agent to induce complete disappearance of tumor in more than one-third of the treated animals. In spite of the marked cytological alterations produced by SK 5357 in the majority of cells of nonresistant S-180, two-thirds of treated tumors continue to grow and their hosts eventually succumb. Such therapeutic failures in the face of pro-

[8] Serial aspirations of iliac crest marrow were made in several dogs anesthetized with sodium thiopental. The authors are indebted to Dr. Leonard Hamilton for his examination of the smears. Two days after initiation of treatment in dogs 1 and 2 (Table 7) toxic cells appeared in both erythroid and myeloid series. There was clumping and condensation of nuclear chromatin with frank pyknosis. These changes persisted through day 11 and were acompanied by apparent, progressive depletion in cellularity. Reduction of erythroid elements appeared greater than of the myeloid series. Samples taken at day 14 showed initiation of recovery with a marked increase in erythrogenesis. Serial aspirations were also studied in two of the four dogs given doses of 10 mg/kg/day. At 11 days after initiation of their treatment abnormalities were seen similar to those described above. Beginning of recovery was noted in samples obtained at 14 days.

[9] Unpublished experiments with D. Lorz.

longed treatment with SK 5357 may be attributed either to the adaptation of partially damaged cells to the agent with subsequent recovery or to the presence, among cells of normal S-180, of a small though significant number of relatively resistant mutants. The present experiments offer no evidence in favor of either possibility, though the latter is consistent with the findings of mercaptopurine-resistant mutants in *L. casei*.

In view of the present observations, 6-mercaptopurine is to be included among the group of chemotherapeutic agents whose effectiveness as curatives may be largely nullified by development of resistance in treated tumors. In the microorganisms drug resistance is accompanied by alterations in purine metabolism and a measurably increased requirement for folic acid (11). If resistance in S-180 is accompanied by similar changes, an increased sensitivity to folic acid antagonists would be expected as a result.

In some respects the toxicology of 6-mercaptopurine resembles that of certain other inhibitors of S-180 such as nitrogen mustards (23), triethylene melamine and other polyethylenimines (25), 4-amino analogs of folic acid (27), and diaminopyrimidine antagonists of folic acid (15). Thus, in mice, rats, and dogs, intoxication with SK 5357 involves progressive weight loss, diarrhea, and lesions in intestinal epithelium as well as hematopoietic damage characterized by reticulocytopenia, leukopenia, and depletion of cellularity in bone marrow. These pathological changes, taken in conjunction with tumor inhibition, suggest a relationship between susceptibility and proliferative activity in different tissues. On the other hand, mercaptopurine differs from the above substances in respect to its hepatotoxicity. (The pulmonary lesions and myocarditis described above in intoxicated rats have been observed only in this species; they will be the subject of a future publication.) Accordingly, the possibility of a relation between hepatic dysfunction and inhibition of S-180 must be considered and is at present under investigation. Such a study is also indicated by recent unpublished observations in this laboratory of other hepatotoxic substances which are inhibitors of S-180, namely, N-methylformamide, as well as active fractions obtained from filtrates of *Aspergillus fumigatus* (28). Furthermore, the latter substances differ from most known inhibitors of S-180 in having little or no deleterious actions in bone marrow and intestinal epithelium.

The results obtained with 6-mercaptopurine have stimulated synthesis and investigation of related purines; these evaluations will be the subject of other publications.

In conclusion, it is to be noted that, again, an agent—6-mercaptopurine—discovered to be an inhibitor of the Crocker mouse sarcoma, has proved active against certain human neoplasms. The compound has been evaluated most particularly in the leukemias, and the results so obtained will be described in another publication.[10]

SUMMARY

6-Mercaptopurine, a purine antagonist for *L. casei*, has been shown to possess unique activity as an inhibitor of the experimental mouse tumor, S-180. The compound prolongs survival time of mice bearing this tumor and, in fact, induces complete recovery in a significant number of animals. These effects may be seen when treatment is initiated either 24 or 96 hours after implantation and when the agent is given by the oral or intraperitoneal route. Cytological disturbance is induced in the tumor to an extent such that the majority of cells lose their characteristic viability when transplanted to normal mice. Finally, a tumor has been developed which demonstrates resistance to 6-mercaptopurine; the implications inherent in this observation may represent a guide in the clinical applications of the agent.

The toxicity of 6-mercaptopurine has been studied in mice, rats, cats, and dogs. No acute deaths were seen. In general, disturbances induced by this agent were referable to the hematopoietic system, the gastrointestinal tract, and the liver. Rats, when exposed to the compound, differed from the other species studied in that myocarditis and pulmonary lesions with effusion developed. 6-Mercaptopurine has been advanced to the stage of clinical evaluation with interesting results.

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REFERENCES

1. BIEBER, S.; NIGRELLI, R. F.; and HITCHINGS, G. H. Effects of Purine and Pyrimidine Analogues on Development of *Rana pipiens*. Proc. Soc. Exper. Biol. & Med., **79**:430–32, 1952.
2. BIESELE, J. J.; BERGER, R. E.; WILSON, A. Y.; HITCHINGS, G. H.; and ELION, G. B. Studies on 2,6-Diaminopurine and Related Substances in Cultures of Embryonic and Sarcomatous Rodent Tissues. Cancer, **4**:186–97, 1951.
3. BUCKLEY, S. M.; STOCK, C. C.; CROSSLEY, M. L.; and RHOADS, C. P. Inhibition of the Crocker Mouse Sarcoma 180 by Certain Ethylenimine Derivatives and Related Compounds. Cancer, **5**:144–52, 1952.

[10] J. H. Burchenal, M. L. Murphy, R. R. Ellison, D. A. Karnofsky, M. P. Sykes, T. C. Tan, L. A. Leone, L. F. Craver, H. W. Dargeon, and C. P. Rhoads, to be published.

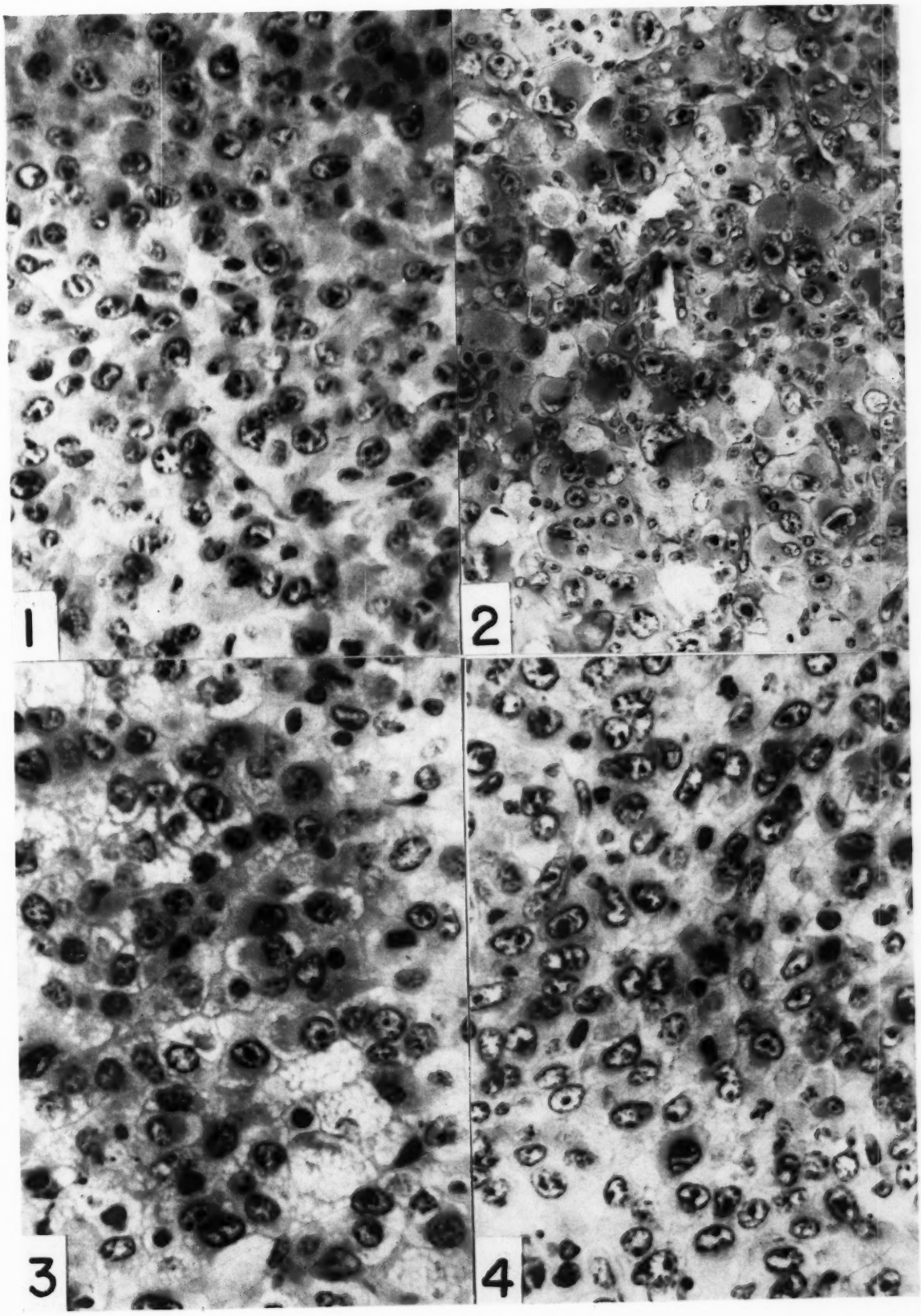
4. Buckley, S. M.; Stock, C. C.; Parker, R. P.; Crossley, M. L.; Kuh, E.; and Seeger, D. R. Inhibition Studies of Some Phosphoramides against Sarcoma 180. Proc. Soc. Exper. Biol & Med., **78**:299–305, 1951.
5. Burchenal, J. H.; Bendich, A.; Brown, G. B.; Elion, G. B.; Hitchings, G. H.; Rhoads, C. P.; and Stock, C. C. Preliminary Studies on the Effect of 2,6-Diaminopurine on Transplanted Mouse Leukemia. Cancer, **2**:119–20, 1949.
6. Cartwright, G. E.; Palmer, J. G.; Hitchings, G. H.; Elion, G. B.; Gunn, F. D.; and Wintrobe, M. M. Studies of the Effect of 2,6-Diaminopurine on the Blood and Bone Marrow of Swine. J. Lab. & Clin. Med., **35**:518–27, 1950.
7. Crossley, M. L.; Allison, J. B.; Wainio, W. W.; and Muenzen, J. B. Chemotherapy of Tumors in Rats. I. The Treatment of Sarcoma 231 in the King A Rat with 2,4,6-Triethylenimino-*s*-triazine and Related Products. J. Nat. Cancer Inst., **12**:305–21, 1951.
8. Elion, G. B.; Burgi, E.; and Hitchings, G. H. Studies on Condensed Pyramidine Systems. IX. The Synthesis of Some 6-Substituted Purines. J. Am. Chem. Soc., **74**:411–14, 1952.
9. Elion, G. B., and Hitchings, G. H. Antagonists of Nucleic Acid Derivatives. IV. Reversal Studies with 2-Aminopurine and 2,6-Diaminopurine. J. Biol. Chem., **187**:511–22, 1950.
10. Elion, G. B.; Hitchings, G. H.; and VanderWerff, H. Antagonists of Nucleic Acid Derivatives. VI. Purines. J. Biol. Chem., **192**:505–18, 1951.
11. Elion, G. B.; Singer, S.; and Hitchings, G. H. The Purine Metabolism of a 6-Mercaptopurine-Resistant *L. casei*. J. Biol. Chem. (in press).
12. Elion, G. B.; Singer, S.; Hitchings, G. H.; Balis, M. E.; and Brown, G. B. Effects of Purine Antagonists on a Diaminopurine-Resistant Strain of *Lactobacillus casei*. J. Biol. Chem., **202**:647–54, 1953.
13. Fisher, R. A. Statistical Methods for Research Workers, pp. 121–27. 10th ed. Edinburgh: Oliver & Boyd, Ltd., 1948.
14. Gellhorn, A.; Engelman, N.; Shapiro, D.; Graff, S,; and Gillespie, H. The Effect of 5-Amino-7-Hydroxy-1-*H*-*v*-Triazolo (*d*) Pyrimidine (Guanazolo) on a Variety of Neoplasms in Experimental Animals. Cancer Research **10**: 170–77, 1950.
15. Hamilton, L.; Philips, F. S.; Clarke, D. A.; Sternberg, S. S.; and Hitchings, G. H. Hematological Effects of Certain 2,4-Diaminopyrimidine Antagonists of Folic Acid. Fed. Proc., **11**:225, 1952.
16. Hertz, R., and Tullner, W. M. Inhibition of Estrogen-induced Growth in the Genital Tract of the Female Chick by a Purine Antagonist; Reversal by Adenine. Science, **109**:539, 1949.
17. Hitchings, G. H.; Elion, G. B.; Falco, E. A.; Russell, P. B.; and VanderWerff, H. Studies on Analogs of Purines and Pyrimidines. Ann. N.Y. Acad. Sc., **52**:1318–35, 1950.
18. Kidder, G. W., and Dewey, V. C. The Biological Activity of Substituted Purines. J. Biol. Chem., **179**:181–87, 1949.
19. Kidder, G. W.; Dewey, V. C.; Parks, R. E., Jr.; and Woodside, G. L. Purine Metabolism in Tetrahymena and Its Relation to Malignant Cells in Mice. Science, **109**: 511–14, 1949.
20. Law, L. W. Studies on the Effect of a Guanine Analog on Acute Lymphoid Leukemias of Mice. Cancer Research, **10**:186–90, 1950.
21. Litchfield, J. T., Jr., and Wilcoxon, F. A Simplified Method of Evaluating Dose-Effect Experiments. J. Pharmacol. & Exper. Therap., **95**:99–113, 1949.
22. Malloy, H. T., and Evelyn, K. A. The Determination of Bilirubin with the Photoelectric Colorimeter. J. Biol. Chem., **119**:481–90, 1937.
23. Philips, F. S. Recent Contributions to the Pharmacology of Bis(2-Haloethyl) Amines and Sulfides. Pharmacol. Rev., **2**:281–323, 1950.
24. Philips, F. S., and Thiersch, J. B. Actions of 2,6-Di. aminopurine in Mice, Rats and Dogs. Proc. Soc. Exper. Biol. & Med., **72**:401–8, 1949.
25. ———. The Nitrogen Mustard-like Actions of 2,4,6-*Tris*-(ethylenimino)-*s*-triazine and other *Bis*(ethylenimines). J. Pharmacol. & Exper. Therap., **100**:398–407, 1950.
26. Philips, F. S.; Thiersch, J. B.; and Bendich, A. Adenine Intoxication in Relation to *in Vivo* Formation and Deposition of 2,8-Dioxyadenine in Renal Tubules. J. Pharmacol. & Exper. Therap., **104**:20–30, 1952.
27. Philips, F. S.; Thiersch, J. B.; and Ferguson, F. C. Studies of the Action of 4-Aminopteroyl-glutamic Acid and Its Congeners in Mammals. Ann. N.Y. Acad. Sc., **52**: 1349–59, 1950.
28. Reilly, H. C., and Stock, C. C. Studies on a Tumor-retarding Agent Produced by *Aspergillus fumigatus*. Cancer Research, **11**:366–69, 1951.
29. Roblin, R. O., Jr.; Lampen, J. O.; English, J. P.; Cole, Q. P.; and Vaughn, J. R., Jr. Studies in Chemotherapy. VIII. Methionine and Purine Antagonists and Their Relation to the Sulfonamides. J. Am. Chem. Soc., **67**: 290–94, 1945.
30. Stock, C. C. Aspects of Approaches in Experimental Cancer Chemotherapy. Am. J. Med., **8**:658–74, 1950.
31. Stock, C. C.; Biesele, J. J.; Burchenal, J. H.; Karnofsky, D. A.; Moore, A. E.; and Sugiura, K. Folic Acid Analogs and Experimental Tumors. Ann. N.Y. Acad. Sc., **52**:1360–78, 1950.
32. Stock, C. C.; Jacobson, W.; and Williamson, M. An Influence of 2,6-Diaminopurine upon the Content of Kappa in *Paramecium aurelia*, Variety 4. Proc. Soc. Exper. Biol. & Med., **78**:874–76, 1951.
33. Sugiura, K.; Hitchings, G. H.; Cavalieri, L. F.; and Stock, C. C. The Effect of 8-Azaguanine on the Growth of Carcinoma, Sarcoma, Osteogenic Sarcoma, Lymphosarcoma and Melanoma in Animals. Cancer Research, **10**:178–85, 1950.
34. Sugiura, K.; Moore, A. E.; and Stock, C. C. The Effects of Aminopterin on The Growth of Carcinoma, Sarcoma and Melanoma in Animals. Cancer, **2**:491–502, 1949.
35. Sugiura, K., and Stock, C. C. Studies in a Tumor Spectrum. I. Comparison of the Action of Methylbis-(2-chloroethyl)amine and 3-Bis(2-chloroethyl)amino-methyl-4-methoxymethyl-5-hydroxy-6-methylpyridine on the Growth of a Variety of Mouse and Rat Tumors. Cancer, **5**:382–402, 1952.
36. ———. Studies in a Tumor Spectrum. II. The Effect of 2,4,6-Triethylenimino-*s*-triazine on the Growth of a Variety of Mouse and Rat Tumors. *Ibid.*, pp. 979–91.
37. Thiersch, J. B., and Philips, F. S. Effects of 4-Amino-Pteroylglutamic Acid in Dogs with Special Reference to Megaloblastosis. Proc. Soc. Exper. Biol. & Med., **71**:484–90, 1949.
38. Wintrobe, M. M. Clinical Hematology, 3d ed. Philadelphia: Lea & Febiger, 1951.

Fig. 1.—Normal S-180. ×400.

Fig. 2.—S-180 following 7 days of treatment with SK 5357, 100 mg/kg. × 400.

Fig. 3.—*Resistant* S-180. From bioassay 7, Chart 3, following 7 days of treatment with SK 5357, 100 mg/kg. ×400.

Fig. 4.—*Resistant* S-180. Control. From bioassay 7, Chart 3. ×400.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Tissue Culture Screening of Amino Acid Analogs for Selective Damage to Mouse Sarcoma Cells*

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INTRODUCTION

The work here reported was undertaken to evaluate the effects of amino acid analogs and various unnatural amino acids on tissue cultures of normal and neoplastic cells. It was hoped that a few of these amino acid analogs would have different effects on normal as compared to neoplastic cells and that we might find some to be considerably more toxic to tumor cells than to normal cells. Assuming that a difference in effects on normal and neoplastic cells implied some difference in metabolism between such cells, an intensive study of these compounds in tissue culture, in conjunction with biochemical studies, might clarify the nature of these differences in metabolism. For a number of phenylalanine antagonists this has actually been done. It has been found that the toxic effect of β-(2-thienyl)-DL-alanine could be blocked by L-phenylalanine in cultures of mouse heart and in cultures of sarcoma T-241, and that phenylpyruvate blocked the toxic effect in heart but not in T-241 cultures. It was found that sarcoma T-241 could not transaminate phenylpyruvate to phenylalanine to any appreciable extent, whereas the heart cultures could. The results on β-(2-thienyl)-DL-alanine have been given in detail in previous papers (10, 11).

METHODS AND MATERIALS

Cultures of heart which contained predominantly fibroblast-like cells, from 1-day- to 2-week-old mice, and cultures of mouse sarcoma T-241 were used throughout.

The method of preparing the chick embryo extract is given in a previous paper (9). Human placental cord serum was employed and was heated for 30 minutes at 60° C. before use. This treatment inhibits most of the clot lysis which usually occurs in the cultures of sarcoma T-241.

Tissue cultures were set up in roller tubes, six implants of heart or of sarcoma T-241 per tube, in a clot made from 1 drop chicken plasma and 1 drop 20 per cent chick embryo extract. The cultures were kept in a rotor run at 10 r.p.h. at 35°–37° C.

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Each culture was fed 0.5 ml. of a mixture of embryo extract, cord serum, and X-6[1] solution (15) in the proportions 2:3:7.5 within 1–2 hours after planting. Twenty-four hours later the compounds to be tested were added in 0.5 ml. X-6 solution. Twenty-four and 48 hours after adding the compounds all cultures were examined and the toxic effects graded from 0–4+. Rounding up of the cells, increase in granularity of the cytoplasm, and disintegration of cells were taken as evidences of a toxic effect. A 4+ toxicity indicates that nearly all cells were rounded up and disintegrating.

Differential toxicity (see Table 1) is defined as the ratio of the concentration of compound giving 2+ toxicity in the heart to the concentration giving a 2+ toxicity in sarcoma T-241 (i.e., a differential toxicity of 4 means that it takes 4 times as much compound to give a 2+ toxicity in heart as in T-241). The inhibition index was determined as follows: Let X be the μM of an antimetabolite with approximately a 4+ toxicity, and Y be the μM of an antimetabolite with approximately a 2+ toxicity. Then, if Z represents the number of μM of metabolite which, when added to X μM of antimetabolite, results in a toxic effect equivalent to that given by Y μM of antimetabolite, the inhibition index $= X - Y / Z$.

It is evident from above that the middle of the toxicity range was used as the end point in the determination of differential toxicity and the inhibition index. This was not always possible, because some compounds were not sufficiently toxic to allow such determinations. In Table 1, these cases are obvious from the toxicity data.

RESULTS

The results are shown in Table 1. For the sake of convenience, the compounds are grouped under various amino acids. This does not imply that they are necessarily antimetabolites for these amino acids. With all compounds, successive concentrations differed by a factor of two. Smaller concentration jumps were usually of little value, because the toxicity grading is rather crude, although one can usually distinguish the difference between the toxic effects of 1½-fold concentration jumps at the middle of the toxicity range. For those compounds in Table 1 for which the concentrations giving a 2+ toxicity are given, two twofold concentration

[1] X-6 solution is made up in double-distilled water in the laboratory to contain the following concentrations of materials, in grams per liter: NaCl, 8.00; KCl, 0.20; $CaCl_2 \cdot 2H_2O$, 0.147; $MgCl_2 \cdot 6H_2O$, 0.203; $NaHCO_3$, 1.010; Na_2HPO_4, 0.213; and glucose, 1.00. The calcium chloride is dissolved separately and added to the solution of the other materials. The solution is then sterilized by filtration.

TABLE 1*

THE TOXICITY OF AMINO ACID ANALOGS TO MOUSE HEART FIBROBLASTS AND CELLS OF SARCOMA T-241 IN TISSUE CULTURE

COMPOUND	Source	CONCENTRATION FOR 2+ TOXICITY AT 48 HOURS: Heart (μM/ml)	T-241 (μM/ml)	Differential Toxicity	Inhibition Index
Tryptophan:					
ethyl-α-nitro-β-(3-indole)-propionate	P	4	4	1	0.2 or less
β-(2-indolyl)-DL-alanine	M	2.5	1.5	2	
4-methyl-DL-tryptophan†	E	(1+ at 10)	10	about 2	
6-methyl-DL-tryptophan	E	10	5	2	2
5-fluoro-DL-tryptophan	E, G	1	0.6	2	4
Cysteine:					
allylglycine	C	30	8	4	
methallylglycine	C	(1+ at 30)	16	about 4	
Methionine:					
methoxinine	I	(1+ at 15)	11	about 4	
ethionine	J	(± at 40)	5	10 or more	
Aspartic acid and asparagine:					
cysteic acid	L	20	5–10	2–4	
α-methyl-aspartic acid	E	(0 at 20)	(0 at 20)		
α-methyl-asparagine	E	(0 at 20)	(0 at 20)		
Phenylalanine:					
β-(2-thienyl)-DL-alanine	B, C	6	5	1	12–24
β-(3-thienyl)-DL-alanine	N	4	3	1	20 or more
β-(2-furyl)-DL-alanine	C	6–8	5	1–2	60
β-(*p*-fluorophenyl)-DL-alanine	B	1	0.5	2	8–16
β-(*o*-fluorophenyl)-DL-alanine	D	2	3	1 or less	30
β-(*m*-fluorophenyl)-DL-alanine	B	1.5	less than 1.5	2 or more	30
β-(*p*-chlorophenyl)-DL-alanine	B	12	6	2	
β-(*o*-chlorophenyl)-DL-alanine	B	6	more than 12	0.5 or less	
β-(*m*-chlorophenyl)-DL-alanine	B	12	less than 3	4 or more	
β-(*p*-bromophenyl)-DL-alanine	B	more than 12	4–5	2–3	
β-(*o*-bromophenyl)-DL-alanine	B	less than 3	more than 12	0.25 or less	
β-(*m*-bromophenyl)-DL-alanine	B	5	3	2	
β-(*m*-iodophenyl)-DL-alanine	B	9	4	2	
β-(*p*-iodophenyl)-DL-alanine‡	K	more than 12	more than 12		
β-(1-naphthyl)-DL-alanine§	C	(± at 9)			
β-(2-naphthyl)-DL-alanine§	M	(± at 9)			
β-(3-thianaphthyl)-DL-alanine	M	3	3	1	
β-(*m*-tolyl)-DL-alanine	O	(1+ at 11)			
β-(*p*-nitrophenyl)-DL-alanine	O	5	5	1	
β-(2-thiazole)-DL-alanine	M	(0 at 12)			
β-(3-pyrazole)-DL-alanine	M	(± at 13)			
β-(2-benzimidazole)-DL-alanine	M	5	2.5	2	
β,β′-(diphenyl)-DL-alanine	O	6	4	1–2	
α-methyl-DL-phenylalanine	E	(± at 11)	(± at 11)		
α-(phenyl)-DL-alanine	O	(± at 20)	(1+ at 20)		
γ-(phenyl)-DL-α-amino-n-butyric acid	A	(± at 20)	(± at 20)		
β-(3,5-dihydroxyphenyl)-DL-alanine	E	10	4	2–3	
N-acetyl-DL-phenylalanine	B	15	15	1	
N-chloroacetyl-DL-phenylalanine	B	4	2	2	
N-dichloroacetyl-DL-phenylalanine	B	15	15	1	
N-carbethoxy-DL-phenylalanine	H	15	4	4	
N-methyl-DL-phenylalanine	A	(± at 20)	20		
N-hydroxyethyl-DL-phenylalanine	B	(0 at 20)	(± −1+ at 20)		
N-(*o*-iodobenzoyl)-DL-phenylalanine	B	7	less than 2.5	more than 3	
N-cyanoethyl-(3,4-dihydroxyphenyl)-DL-alanine	E	2.5	less than 1.25	more than 2	
Others:					
aminomethane sulfonic acid	A	less than 2.5	2.5	less than 1	
DL-norvaline	K	(± at 40)	30		
methionine sulfoxide	F	(± at 11)	(± at 11)	about 1	

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† Not all dissolved at 10 μM/ml.

‡ All not in solution at 12 μM/ml.

§ Insoluble at higher concentrations.

jumps on either side of this will cover the toxicity range 0–4+.

For compounds of low toxicity, the data are from one to two experiments only. For compounds which seemed of special interest to us, such as β-(2-thienyl)-DL-alanine, ethionine, β-(2-indolyl)-DL-alanine, and the halogenated phenylalanines, the toxicity data may come from more than twenty experiments, since a toxicity experiment was always run concomitantly with each blocking experiment. All inhibition indices come from at least two experiments. Nonetheless, the figures should be considered as approximate only. The toxicity grading method is not very sensitive, and one cannot accept the data in Table 1 as being on the same level of accuracy as comparable data from experiments with bacteria. This is not entirely the fault of the toxicity grading method but is partly owing to the inherent variations in the complex medium used in tissue culture work. This is particularly important when testing antimetabolites that have a high inhibition index. For these, slight variations in the amount of normal metabolite present in the medium could shift the toxicity range considerably. There seems to be no way to obviate this variability at present. We must wait for fully defined tissue culture media before we can expect more accurate results. Taking these factors into account, the differential toxicity and inhibition index may vary by a factor of two under the average conditions in our laboratory.

The inhibition indices given in Table 1 are from experiments in which the antimetabolite was tested against the normal amino acid under which it is listed. However, the blocking experiments were more extensive than the table indicates. Additional blocking experiments are summarized below.

The toxicity of β-(2-indolyl)-DL-alanine was not blocked by L-tryptophan even when the cultures were pretreated for 24 hours with twice as much L-tryptophan as indolylalanine. L-Phenylalanine and glycine seemed to slow the onset of toxicity. The following compounds had no effect on the toxicity of β-(2-indolyl)-DL alanine: L-histidine, DL-alanine, DL-threonine, DL-valine, L-leucine, DL-isoleucine, DL-norleucine, DL-aspartic acid, L-glutamic acid, L-lysine, L-arginine, L-cystine, L-cysteine, L-methionine, L-tyrosine, L-proline, L-hydroxyproline, β-(2-benzimidazole)-DL-alanine, benzimidazole, adenine, guanine, xanthine, hypoxanthine, uracil, thymine, cytosine, nicotinic acid, thiamin, pyridoxine, panthotenate, riboflavin, *p*-aminobenzoic acid, inositol, ascorbic acid, and folic acid.

Ethionine toxicity was not blocked by L-methionine, L-cystine, L-cysteine, DL-homocystine, DL-homocysteine, choline, creatine, glutathione, guanidoacetic acid, or an equimolar mixture of glycine and arginine. Methionine, homocysteine, homocystine, and cysteine gave about the same toxicity picture as ethionine, at about the same molar concentrations.

The inhibition index of β-(2-thienyl)-DL-alanine antagonized by L-phenylalanine was usually somewhat higher for heart than for T-241. Phenylpyruvate blocked toxicity of the compound in heart cultures but not in T-241 cultures. None of the following compounds had any effect on the toxicity of β-(2-thienyl)-DL-alanine: L-tyrosine, L-tryptophan, D-phenylalanine, phenyllactic acid, phenylacetic acid, L-leucine, DL-isoleucine, DL-norleucine, DL-alanine, glycine, DL-threonine, L-cystine, L-cysteine, DL-aspartic acid, L-glutamic acid, DL-valine, L-histidine, L-arginine, L-lysine, glucose, acetoacetic acid, pyruvic acid, and α-ketoglutaric acid.

With β-(3-thienyl)-DL-alanine, it was found that phenylpyruvate blocked toxicity in heart cultures but not in T-241 cultures. Tyrosine did not block the toxicity. Similar results with phenylpyruvate and tyrosine were found with β-(2-furyl)-DL-alanine.

The inhibition index of β-(*p*-fluorophenyl)-DL-alanine, an antagonist of L-phenylalanine, was usually somewhat higher for heart than for T-241. Phenylpyruvate blocked toxicity in heart cultures but not in T-241 cultures. Tyrosine did not block the toxicity of β-(*p*-fluorophenyl)-DL-alanine.

Phenylpyruvate blocked the toxicity of β-(*o*-fluorophenyl)-DL-alanine and of β-(*m*-fluorophenyl)-DL-alanine in heart cultures, but not in T-241 cultures. Tyrosine did not block their toxicity.

The toxicity of β-(*p*-chlorophenyl)-DL-alanine was not blocked by L-phenylalanine, L-tyrosine, or phenylpyruvate. These three compounds were also ineffective against each of the following: β-(*o*-chlorophenyl)-DL-alanine, β-(*m*-chlorophenyl)-DL-alanine, β-(*p*-bromophenyl)-DL-alanine, β-(*m*-bromophenyl)-DL-alanine, β-(*o*-bromophenyl)-DL-alanine, β-(*m*-iodophenyl)-DL-alanine, and β-(3-thianaphthyl)-DL-alanine. Nor was the toxicity of β,β′-(diphenyl)-DL-alanine blocked by L-phenylalanine or phenylpyruvate.

The relatively nontoxic α-methyl-DL-phenylaline did not block the toxic effects of β-(2-thienyl)-DL-alanine.

DISCUSSION

The results with amino acid analogs may be compared to the corresponding data on purine

analogs obtained by Biesele and his co-workers at this institute (4–7). The most toxic of the amino acid analogs tested so far are less toxic than the most toxic purine analogs examined by Biesele *et al.* In general, the concentrations of purine analogs giving median toxicities are somewhat lower than the corresponding concentrations of the group of amino acid analogs. Some of the purine analogs have been shown to act as anti-adenines. There is good reason to think that many of the amino acid analogs interfere with protein synthesis. This may suggest that the mammalian cells studied are more sensitive to interferences in nucleic acid metabolism than to interferences with protein metabolism. This would certainly be commensurate with the central roles in protein synthesis now attributed to the nucleic acids. However, the possibility must also be entertained that the ill-defined tissue culture media may contain an effectively higher level of substances tending to antagonize the amino acid analogs than of material tending to counteract the purine analogs.

Not only were the purine analogs generally more toxic than the amino acid analogs, but for those compounds which gave good differential toxicities, these were considerably higher than has been found with the amino acid analogs. It is apparent from Biesele's results and from ours that low differential toxicities of the order of 2 are fairly common. We do not consider these as evidence of any specific effect on the tumor cells. From our general experience in toxicity studies on tumor and normal cells in tissue culture, it seems that cells of many tumors are more sensitive than normal cells to various deleterious influences.

Our results suggest some generalizations as to the efficacy of various substitutions in making antimetabolites. We have had poor results with compounds in which a methyl group was substituted for a hydrogen, whether on a ring system or otherwise. Most of these compounds were not toxic enough for us to determine inhibition indices. On the other hand, a halogen substituted for a hydrogen on a ring has always given fairly toxic compounds, although only the fluorine-substituted compounds acted as competitive antagonists. This is shown by the group of ring-substituted, monohalogenated phenylalanines. The monofluoro phenylalanines were all good competitive antagonists, the inhibition indices being considerably higher than have been found for *L. arabinosus* (3) and *Neurospora crassa* (13). The fluorophenylalanines have also been shown to act as antiphenylalanines in experiments on rat growth, although in the intact rat there is some toxicity due to the production of inorganic fluoride which cannot be blocked by phenylalanine (1, 2). There may be some such effect in tissue culture also, since we have not always been able to block the toxic effect completely with L-phenylalanine; in any case, this effect has not been prominent in the tissue culture studies. Although only the fluorophenylalanines acted as competitive antagonists, there is an interesting correlation between the relative differential toxicities of the para, meta, and ortho substituted fluoro-, chloro-, and bromo-phenylalanines. In each case, the para and meta substituted compounds were more toxic to T-241 than to heart cultures, but the ortho substituted phenylalanines were more toxic to heart than to T-241 cultures, the effect increasing in the order F, Cl, Br. The para and meta halogen-substituted phenylalanines were more alike, and the ortho substituted phenylalanines seemed different. This suggests that whatever the basis for these differential toxicities, it depends on a steric effect and does not depend on a reaction with the ring. Such steric effects are not uncommon in biological systems. For example, the degree of cross reaction observed between ortho, meta, and para substituted benzene ring haptens depends primarily on the steric factor (14). If a reaction with the ring were involved one might expect the usual ring substitution reactivity series for the benzene nucleus (*i.e.*, ortho and para alike and meta different). From the similarity in differential toxicities, it is possible that the chloro- and bromo-substituted phenylalanines interfere with the same system as the fluoro-substituted phenylalanines, although noncompetitively. This is probably an interference with protein synthesis. The fluoro-phenylalanines are similar to β-(2-thienyl)-DL-alanine, β-(3-thienyl)-DL-alanine, and β-(2-furyl)-DL-alanine (10). With all these compounds there is an essentially competitive antagonism with L-phenylalanine. Phenylpyruvate can replace L-phenylalanine in heart cultures but not in sarcoma T-241 cultures, because the heart cultures can transaminate phenylpyruvate to form L-phenylalanine, whereas the T-241 cultures cannot do so. It is apparent that the thiophene and furan rings are rather good substitutes for the benzene ring but that naphthyl, thianaphthyl, thiazole, and pyrazole rings are poor substitutes for the benzene ring. The above results on mammalian cells in tissue culture fit in quite well with the generalization that effective competitive antagonists do not differ much in structure from their metabolite and that large structural changes result either in lowered activity or in a compound which acts as a noncompetitive antagonist (20).

Two other compounds have been of special

interest to us, β-(2-indolyl)-DL-alanine and ethionine, the former because of its rather high toxicity and the latter because of its high differential toxicity. The shift of the alanine side chain from the 3- to the 2-position in tryptophan involves a considerable change in structure. L-Phenylalanine and glycine seemed to slow down the rate of appearance of toxicity, but no real blocking agents were found. It is possible that a mixture of amino acids would have some effect. Ethionine represents a special case: Its over-all toxicity is low, but it has a high differential toxicity. Although in one experiment there was slight evidence for a blocking effect with methionine, this could not be repeated. In all subsequent experiments, methionine increased the toxicity of ethionine. Furthermore, methionine, homocysteine, homocystine, and cysteine were found to give about the same toxicity pictures as ethionine. In the rat and mouse the toxicity of ethionine can be blocked by methionine and choline (16, 19), but even in these animals the picture is not one of simple competitive antagonism. There is evidence that some of the ethionine toxicity in the rat is due to: (*a*) transfer of the ethyl group to form toxic ethyl analogs of normal metabolites such as choline (17, 18) and, possibly, (*b*) some incorporation of ethionine into the body proteins to give abnormal proteins (12). The action of ethionine in tissue cultures is probably as complex as in the intact animal. The toxicity picture seems to be due to a property common to ethionine and methionine; it does not seem to be related to transmethylation.

Most of the compounds in Table 1 have been tested against Crocker mouse sarcoma 180 in the *in vivo* screening program at the Sloan-Kettering Institute and have given negative results.

SUMMARY

1. Comparative toxicities in tissue cultures of mouse heart and of sarcoma T-241 are reported for 50 amino acid analogs.

2. Although highly toxic compounds were found in this group, high selective toxicities to sarcoma T-241 were rare.

3. Of a group of halogenated phenylalanines, only the fluorophenylalanines were competitive antagonists of L-phenylalanine, but the effect of the position of substitution on the differential toxicity was similar for the chloro- and bromophenylalanines.

4. Ethionine showed a fairly high selective toxicity to sarcoma T-241. Its toxicity was not blocked by methionine or choline. Methionine gave about the same toxicity picture as did ethionine.

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REFERENCES

1. ARMSTRONG, M. D., and LEWIS, J. D. The Toxicity of *o*- and *p*-Fluorophenyl-DL-alanines for the Rat. J. Biol. Chem., **188**:91–95, 1951.
2. ———. Effect of Phenylalanine on the Toxicity of 4-Fluorophenylalanine for the Rat. *Ibid.*, **190**:461–66, 1951.
3. ATKINSON, E. E.; MELVIN, S.; and FOX, S. W. Effects of *p*-Fluorophenylalanine on the Growth of *Lactobacillus arabinosus*. Arch. Biochem. & Biophys., **31**:205–11, 1951.
4. BIESELE, J. J. Purine Antagonism and Differential Toxicity of Some Imidazo-1,2,3-triazines in Mouse-tumor Tissue Cultures. Cancer, **5**:787–91, 1952.
5. BIESELE, J. J.; BERGER, R. E.; and CLARKE, M. Tissue Culture Screening of Purines and Purine Nucleosides for Selective Damage to Mouse Sarcoma Cells. Cancer Research, **12**:399–406, 1952.
6. ———. Antagonism Studies with Several Toxic Purines and Purine Nucleosides in Mouse Tissue Cultures. *Ibid.*, pp. 465–70.
7. BIESELE, J. J.; BERGER, R. E.; WILSON, A. Y.; HITCHINGS, G. H.; and ELION, G. B. Studies on 2,6-Diaminopurine and Related Substances in Cultures of Embryonic and Sarcomatous Rodent Tissues. Cancer, **4**:186–97, 1951.
8. HARDIN, J. O., and HOVE, E. L. Prevention of DL-Methionine Toxicity in Rats by Vitamins E, B_{12}, Folacin, Glycine, and Arginine. Proc. Soc. Exper. Biol. & Med., **78**:728–31, 1951.
9. JACQUEZ, J. A., and BARRY, E. Tissue Culture Media. The Essential Non-dialyzable Factors in Human Placental Cord Serum. J. Gen. Physiol., **34**:765–74, 1951.
10. JACQUEZ, J. A.; BARCLAY, R. K.; and STOCK, C. C. Transamination in the Metabolism of β-2-Thienyl-DL-alanine in Normal and Neoplastic Cells *in Vitro*. J. Exper. Med., **96**:499–512, 1952.
11. JACQUEZ, J. A.; STOCK, C. C.; and BARCLAY, R. K. Effect of β-2-Thienyl-DL-alanine on the Growth of Sarcoma T-241 in C-57 Black Mice. Cancer (in press).
12. LEVINE, M., and TARVER, H. Studies on Ethionine. III. Incorporation of Ethionine into Rat Proteins. J. Biol. Chem., **192**:835–50, 1951.
13. MITCHELL, H. K., and NIEMANN, C. The Competitive Inhibition of the Metabolism of α-Amino Acids by Their Halogenated Analogs. J. Am. Chem. Soc., **69**:1232, 1947.
14. PAULING, L., and PRESSMAN, D. The Serological Properties of Simple Substances. IX. Hapten Inhibition of Precipitation of Antisera Homologous to the *o*-, *m*-, and *p*-Azophenylarsonic Acid Groups. J. Am. Chem. Soc., **67**:1003–12, 1945.
15. SIMMS, H. S., and SAUNDERS, M. Use of Serum Ultrafiltrate in Tissue Cultures for Studying Deposition of Fat and for Propagation of Viruses. Arch. Path., **33**:619–35, 1942.
16. STEKOL, J. A., and WEISS, K. A Study on Growth Inhibition by D-, L-, and DL-Ethionine in the Rat and Its Alleviation by the Sulfur-containing Amino Acids and Choline. J. Biol. Chem., **179**:1049–56, 1949.
17. ———. On Deethylation of Ethionine in the Rat. *Ibid.*, **185**:577–83, 1950.
18. ———. The Inhibition of Growth of Rats by Triethylcholine. *Ibid.*, pp. 585–87.
19. ———. Inhibition of Growth of C3H Mice by Ethionine and Its Alleviation by Methionine or Choline. Proc. Soc. Exper. Biol. & Med., **77**:213–14, 1951.
20. WOOLLEY, D. W. A Study of Antimetabolites. New York: John Wiley & Sons, Inc., 1952.

The Heterologous Transplantation of the V-2 Rabbit Carcinoma*

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One of the characteristic properties of the Shope papilloma which differentiates it from other tumors in premalignant phases of development is its ability to survive and grow on heterologous transfer (4). This property is present from inception and appears to derive from the intracellular location of the virus and its necessarily concomitant passage to the new host. The final developmental stage of the papilloma is carcinoma, and at this stage the tumor is autonomous and no longer depends for its existence on the continued presence of the virus. The V-2 carcinoma represents this stage of papilloma development (6–8), and a study of its transplantation reactions was undertaken to provide a basis for comparison with its precursor, the virus-dependent papilloma.

MATERIALS AND METHODS

The V-2 carcinoma was obtained as an intramuscular growth in a rabbit, through the courtesy of Dr. John Kidd. The tumor was transferred to the brains of other rabbits and carried by serial passage in this site. Brain transplants are solid, cellular, and free of the desmoplastic connective tissue and necrosis characteristic of growths in the muscle or testicle. The tumor tissue used for heterologous transfer was derived from fresh, growing brain transplants.

The alien species tested included the rat, mouse, guinea pig, and hamster. The mice were DBA's, bred from Bar Harbor stock, and the rats were of Sprague-Dawley strain. The hamsters were obtained from Tumblebrook Farm of Brant Lake, New York, and the guinea pigs from a local breeder. The animals were not selected for age or sex.

The eye, brain, testicle, and subcutaneous space were used as sites for transplantation. Whole fragments of tissue rather than cellular suspensions were employed for transfer, and all transfers were made by means of a trocar. The special technics utilized in transplantation to the anterior chamber and brain have been described (2, 3).

RESULTS

The transplantability of the V-2 carcinoma was tested in the eye, brain, subcutaneous space, and testicle of guinea pigs and in the three latter sites in mice, hamsters, and rats. Transfer was successfully effected in the guinea pig's eye and in the brain of all four species, but takes in the subcutaneous space and testicle were obtained only in hamsters and mice.

Guinea pigs.—Transfer to the guinea pig's eye was successful in all the ten animals used. Growth was apparent by the 4th day, and the anterior chamber was filled with tumor by the 15th day. Thereafter, the transplant underwent rapid regression, and no living tumor cells were found at the end of 3 weeks. Histologically, the growing tumor was made up of solid masses of epidermoid cells with little necrosis and no desmoplastic reaction (Fig. 1).

Growth in the brain was obtained in 36 of the 40 guinea pigs used, and the tumor has been carried in this site for seven consecutive passages. Growth is rapid, and the tumor attains a mass measuring 1 cm. in diameter during the 3d week. Neurological signs of increased intracranial pressure develop near the end of the 3d week, and their appearance is rapidly followed by death of the animal.

The tumor mass is only a shade darker in color than the white matter of the brain, and its boundaries are not readily distinguishable, but it is easily limited from the surrounding tissue by its increased firmness, and a comparatively clean dissection is possible. The histological picture is similar to that found in tumor transplanted to the eye. Some tumors contain irregular zones of necrosis or hemorrhage, but the majority consist almost exclusively of intact cells arranged in an epidermoid pattern and supplied with a scanty but well vascularized stroma (Figs. 2–4). The growths are less well defined than is suggested by gross examination, and microscopic tongues of tumor tissue extend beyond the main mass into the adjacent brain.

A series of twenty guinea pigs was used for subcutaneous transfer of the tumor and another series of twenty for testicular transfer. The animals were held under observation for 2 months,

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but in no instance was there gross or microscopic evidence of growth.

Mice.—Transplantation of the V-2 carcinoma to the mouse brain is almost invariably successful, and takes have occurred in 90 per cent of the 140 mice used. Serial transfer is readily effected and offers an economical means of carrying the tumor for stock purposes. As a rule the animals die or are discovered with neurological signs about the 20th day after transfer, and at autopsy the brains contain tumors replacing a quarter to a half of a cerebral hemisphere. Microscopically, such tumors show fewer degenerating cells than are characteristic of guinea pig growths, but they are otherwise identical (Figs. 5, 6). Occasionally, mice survive for a month or more, and in such instances large areas of the tumor are necrotic, but living cells persist in perivascular foci.

Subcutaneous transfer is successful in approximately 50 per cent of mice, and the source of the tumor, whether from the rabbit or mouse brain or subcutaneous space of another mouse, appears to be without influence in this respect. The growing transplant is usually palpable by the 10th day, but sometimes takes are not evident in less than a month. Subsequent growth is slow and progressive, and regression has not been observed, although tumor-bearing animals have been held for as long as 140 days. During this period the tumors continue to increase in size and in several instances have attained a bulk approximately one-half that of the host (Figs. 7, 8). On section, such tumors contain a large amount of necrotic material, but the peripheral zone is thick and fleshy and microscopically is made up of living tumor cells (Figs. 9, 10). Neither lymphatic extension nor metastasis has been observed.

Transplantation to the mouse testicle is generally successful, but regression begins about the time the growing tumor becomes palpable, and histological section on the 10th day shows large areas of necrosis with only isolated marginal groups of living cells.

Hamsters.—Transplantation to the hamster's brain has resulted in takes in all but two of the 68 animals used, and the tumor has been maintained by serial transfer for seven consecutive generations. As in other species, the appearance of neurological signs is followed shortly by death of the animal near the end of the 3d week after transfer. At autopsy the tumors are larger in relation to brain size than those found in the guinea pig or mouse, and they are generally softer in consistency. Microscopically, they show no feature distinguishing them from analogous growths in other species (Figs. 11, 12).

The incidence of takes in the subcutaneous space of the hamster is as high as that noted in the brain, and it seems probable that the few failures in either site arise from errors in transplantation technic. The growing tumor may attain a palpable size in 10 days, but not infrequently takes cannot be detected before the 3d or 4th week. A sudden regression of small tumors occasionally occurs, but as a rule growth continues, and the tumors reach a bulk comparable in relation to body size to those found in the mouse (Fig. 13). In contrast to the mouse transplants, however, all the large hamster tumors observed have begun to regress at about the 125th day, and in several instances have completely disappeared. Young tumors are medullary in character and consist of solid masses of epidermoid cells without necrosis or desmoplasia. In contrast, old tumors contain large amounts of necrotic material, but as late as the 120th day the bulk of the growth is made up of healthy-appearing intact cells (Fig. 14). Neither lymphatic extension nor metastasis has been observed.

Transfer to the hamster's testicle results in a large percentage of takes, but growth of the transplant is associated with inflammatory reaction, and fibrosis and regression invariably occur before the 50th day (Fig. 15).

Rat.—The results of transfer to the rat's brain are not different from those obtained at the same site in other species. A large percentage of takes occur, and the growths reach sufficient size to kill the animals about the 20th day. Attempts to transfer to the subcutaneous space and testicle have not been successful.

DISCUSSION

The wide transplantability range of the V-2 carcinoma simulates that of its precursor, the Shope papilloma. Like the V-2 tumor, the papilloma grows on transfer to the brains of guinea pigs, hamsters, rats, and mice and to the subcutaneous space of hamsters and mice (4, 5). This is a unique situation among tumors, for in other instances transplantability during premalignant phases of development is sharply limited to animals of a specific constitutional status; while, in the Shope papilloma, the conditioning factor is resident in the tumor cell rather than in the host and necessarily accompanies the tumor cell on transfer. The V-2 carcinoma represents a late developmental stage of the papilloma, characterized biologically by independence of the conditioning factor (6–8) and the ability to metastasize. Its heterologous transplantability is thus an expression of its autonomy and is not, as in the case of the papilloma, dependent on the coincident

transfer of the virus. The parallel transplantability of the V-2 carcinoma and the Shope papilloma presents an unusual material for investigation, and, by virtue of the fact that both premalignant and malignant phases of the development of the same tumor can be studied simultaneously in the same host or in widely divergent species, provides an approach to a number of questions of concern to cancer research in general as well as to specific problems associated with the papilloma-carcinoma sequence.

The ability of the V-2 carcinoma to grow on subcutaneous transfer to hamsters and mice without previous passage through the eye or brain of the alien species is shared by the Brown-Pearce carcinoma but is not a property of other tumors studied (1). The heterologous subcutaneous transfer of other tumors cannot be effected with tissue derived directly from the foreign species, although growth in the eye or brain is usual under such circumstances. In the present instance, however, the subcutaneous space proved as effective a nidus for growth of heterologous fragments as did the brain. This was particularly true in the hamster and reflects other experience with this species. Apparently, the hamster's subcutaneous connective tissue is unusually responsive to stroma-inducing stimuli and proliferates to provide a scaffolding and blood supply for transplants of tissue that, in other species, provoke no reaction or act as foreign bodies and elicit an inflammatory response. In any case, takes occur in a high percentage of cases, and the ensuing growths are progressive for long periods of time.

REFERENCES

1. Greene, H. S. N. Heterologous Transplantation of the Brown-Pearce Tumor. Cancer Research, **9**:728–35, 1949.
2. ———. The Heterologous Transplantation of Human Melanomas. Yale J. Biol. & Med., **22**:611–20, 1950.
3. ———. The Transplantation of Tumors to the Brains of Heterologous Species. Cancer Research, **11**:529–34, 1951.
4. ———. The Induction of the Shope Papilloma in Transplants of Embryonic Rabbit Skin. *Ibid.*, **13**:58–63, 1953.
5. ———. The Induction of the Shope Papilloma in Homologous Transplants of Embryonic Rat Skin. *Ibid.* (in press).
6. Smith, W. E.; Kidd, J. G.; and Rous, P. Recovery and Disappearance of the Rabbit Papilloma Virus (Shope) from the Carcinomas That Originate from Papilloma Cells. Fourth International Cancer Congress, **84**:2, 1947.
7. Syverton, J. T.; Dascomb, H. E.; Wells, E. B.; Koomen, J.; and Berry, G. P. The Virus-induced Rabbit Papilloma-to-Carcinoma Sequence. II. Carcinomas in the Natural Host, the Cottontail Rabbit. Cancer Research, **10**:440–44, 1950.
8. Syverton, J. T.; Wells, E. G.; Koomen, J.; Dascomb, H. E.; and Berry, G. P. The Virus-induced Papilloma-to-Carcinoma Sequence. III. Immunological Tests for Papilloma Virus in Cottontail Carcinomas. Cancer Research, **10**:474–82, 1950.

Fig. 1.—Transplant of V-2 carcinoma in anterior chamber of guinea pig's eye. The animal was killed 2 weeks after transfer. ×9.

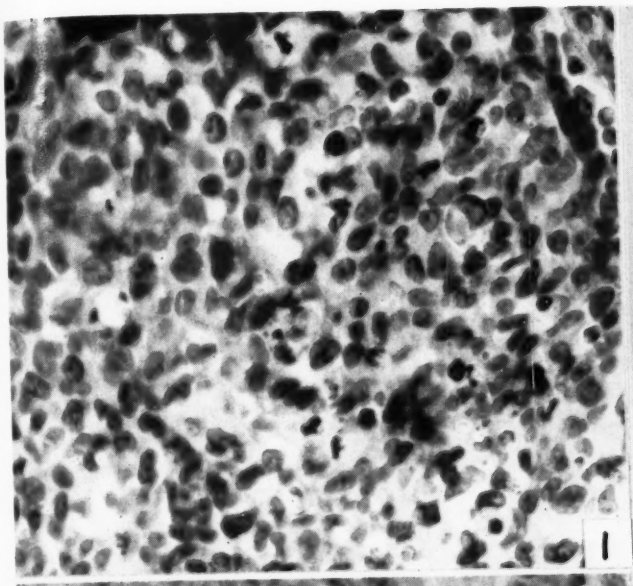
Fig. 2.—Guinea pig brain bearing transplant of V-2 carcinoma 3 weeks after transfer. ×9.

Fig. 3.—Transplant of V-2 carcinoma in guinea pig's brain 20 days after transfer. ×500.

Fig. 4.—Edge of V-2 carcinoma growing in guinea pig's brain. Note absence of a circumscribed boundary between tumor and brain. ×500.

Fig. 5.—Edge of V-2 carcinoma growing in mouse brain. Note circumscribed boundary. ×500.

Fig. 6.—Transplant of V-2 carcinoma in mouse brain 20 days after transfer. ×500.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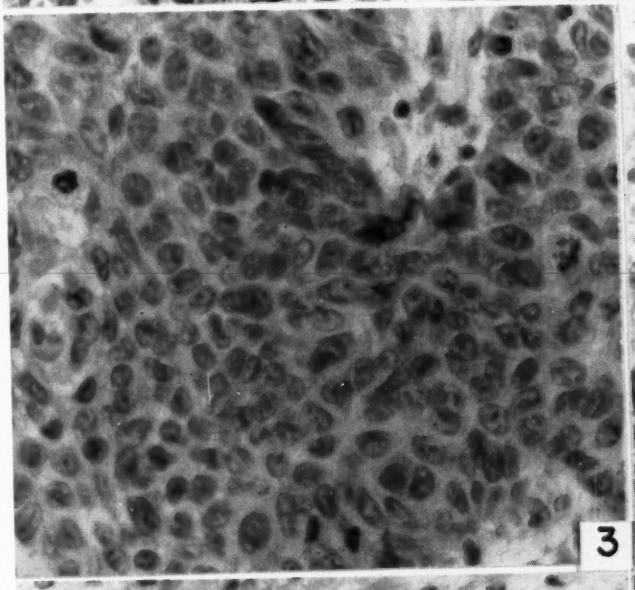
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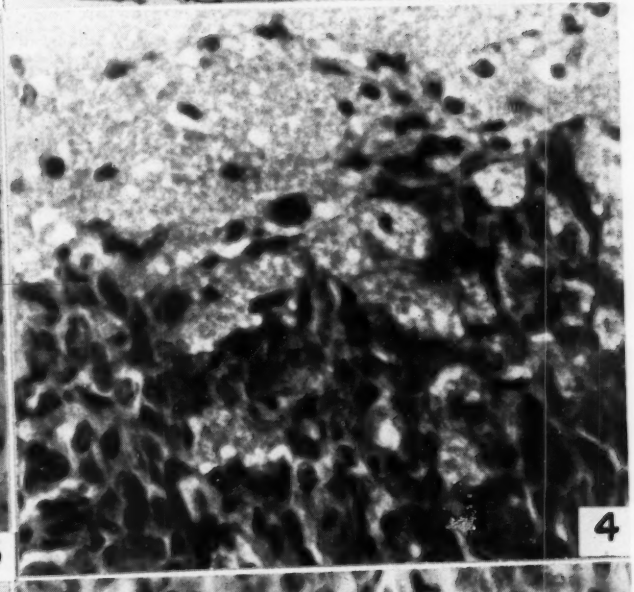
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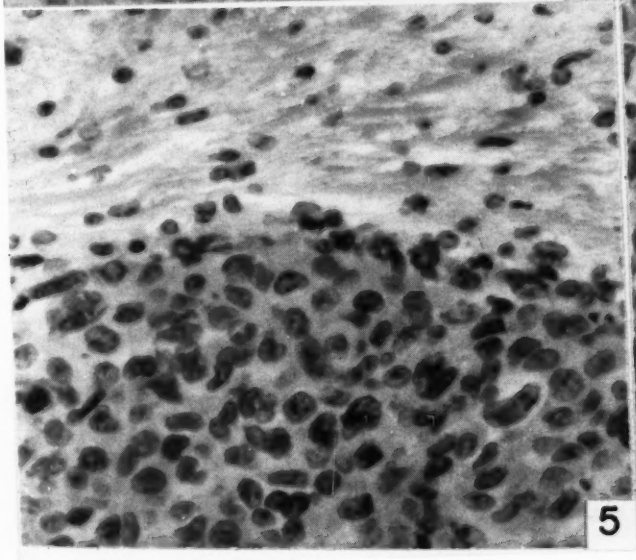
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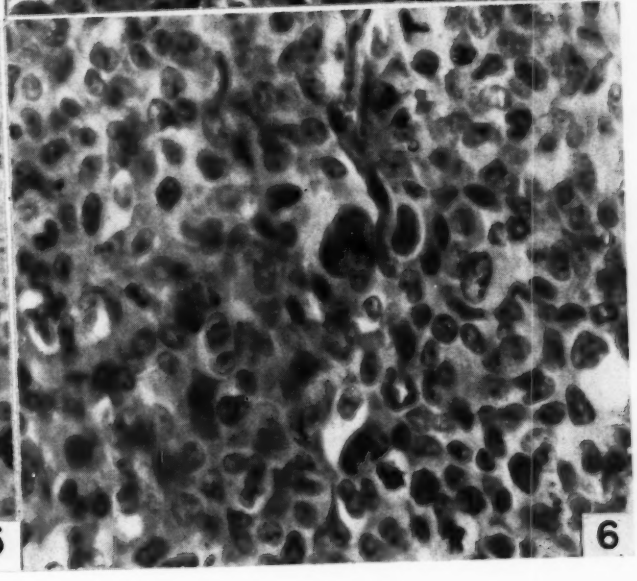
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FIG. 7.—Transplant of V-2 carcinoma in subcutaneous space of a DBA mouse 80 days after transfer.

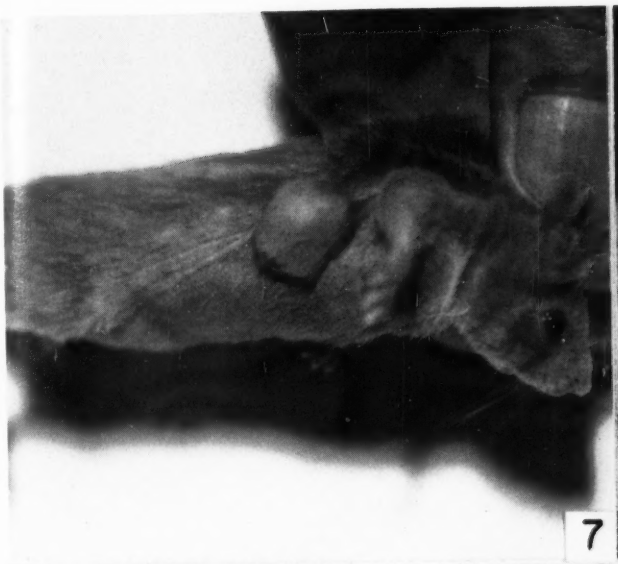
FIG. 8.—Transplant of V-2 carcinoma in subcutaneous space of a DBA mouse 140 days after transfer.

FIG. 9.—Transplant of V-2 carcinoma in subcutaneous space of a mouse 75 days after transfer. ×500.

FIG. 10.—Transplant of V-2 carcinoma in subcutaneous space of a mouse 135 days after transfer. The cells are pale and vacuolated but are viable and survive serial transfer. ×500.

FIG. 11.—Transplant of V-2 carcinoma in brain of a hamster 20 days after transfer. ×9.

FIG. 12.—Higher power view of tumor shown in Figure 11. ×500.



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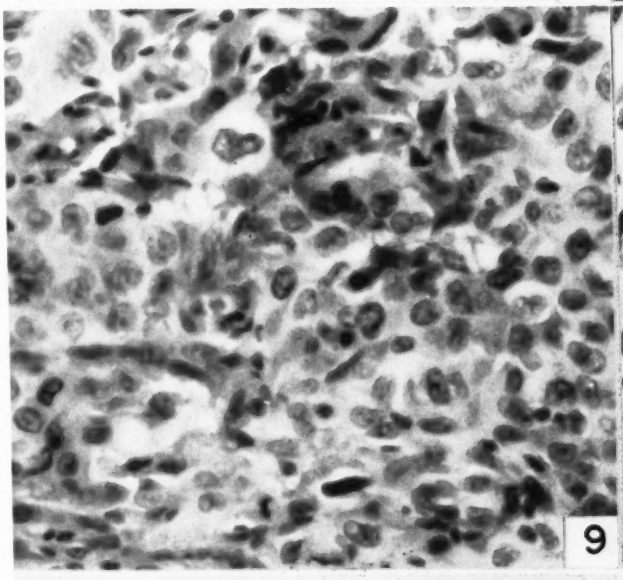

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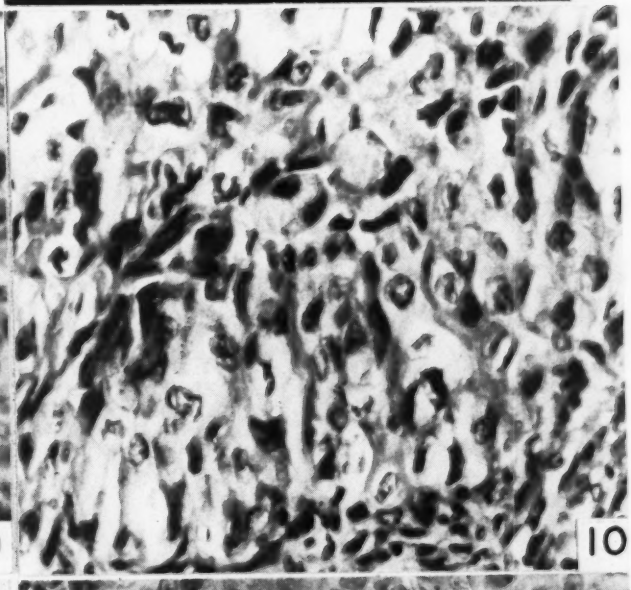

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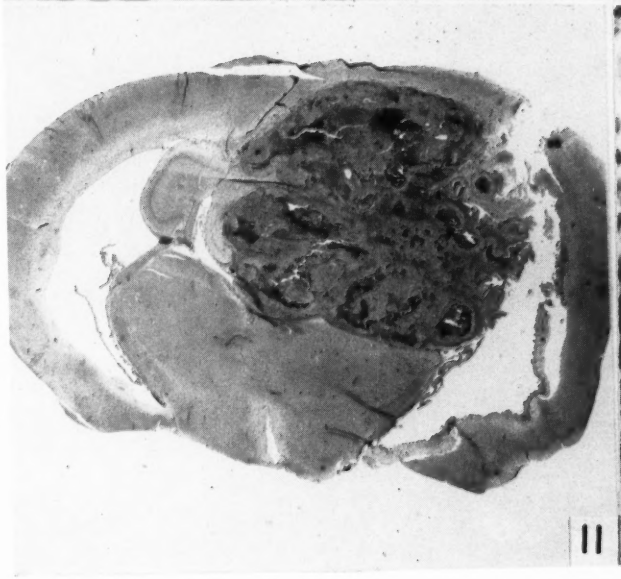

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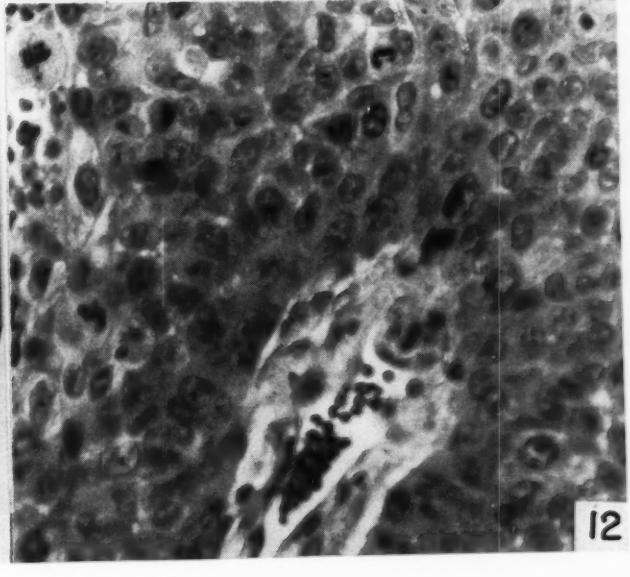

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FIG. 13.—Transplant of V-2 carcinoma in subcutaneous space of a hamster 120 days after transfer.

FIG. 14.—Section of transplant shown in Figure 13. ×500.

FIG. 15.—Transplant of V-2 carcinoma in hamster's testicle 31 days after transfer. ×500.

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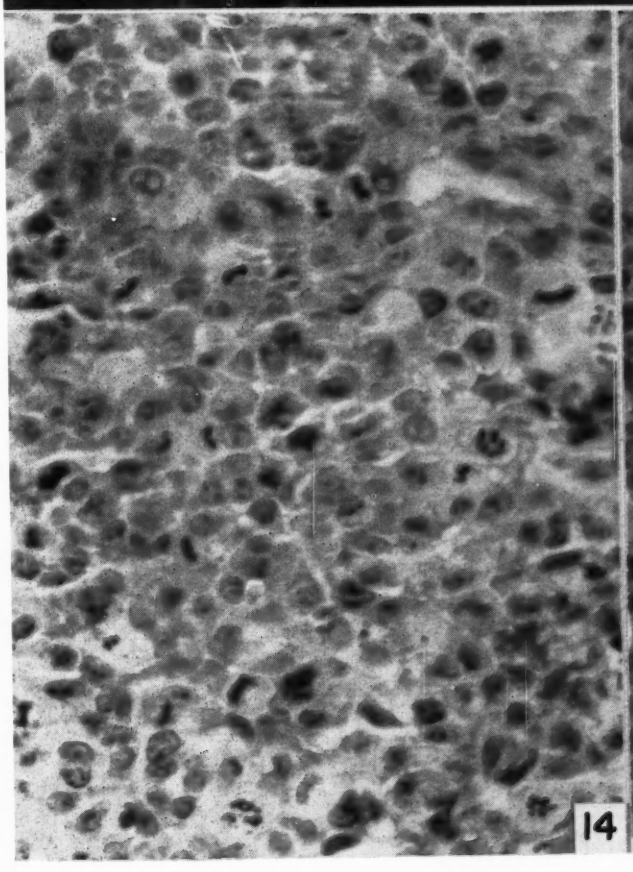

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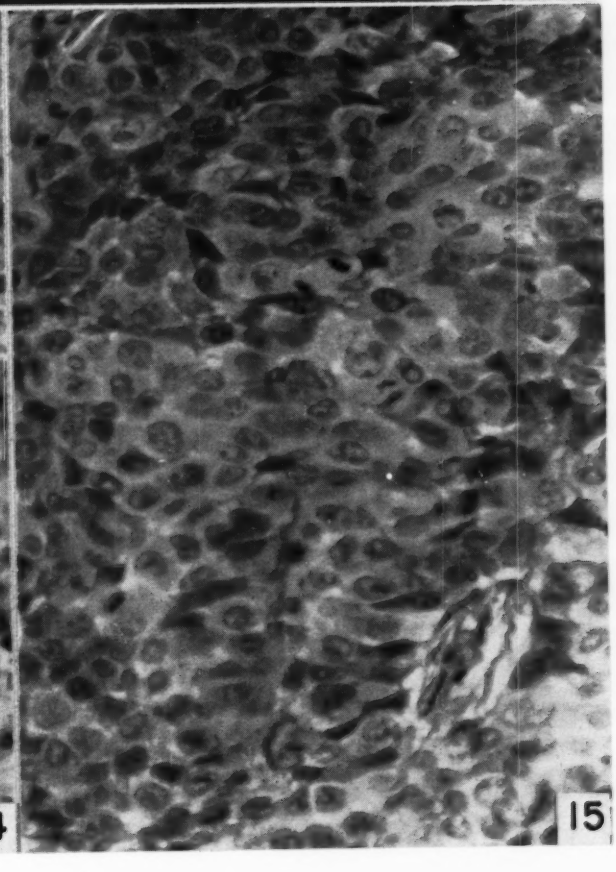

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Book Reviews

Atlas of Tumor Pathology. By 38 or more authors, in 24 volumes (40 fascicles). Published under the Auspices of the Subcommittee on Oncology, Committee on Pathology, National Research Council, by The Armed Forces Institute of Pathology, American Registry of Pathology, 7th and Independence Avenue, Washington 25, D.C.

A mere listing of the titles and authors of the eleven volumes (fourteen fascicles) already in print should be indication enough of the high quality of experience and scholarship collaborating to make this Atlas, and also of the sound scheme and large scope of its final whole. The "Introduction and Table Contents" (Section I. Fascicle 1), by BALDUIN LUCKÉ, succinctly describes the over-all plan: Sections dealing with tumors (I) of the Skin and Cutaneous Melanomas; (II) of the Musculo-skeletal System; (III) the Cardiovascular and Hematopoietic Systems; (IV) Oral Cavity, Upper Respiratory Tract, Neck and Ear; (V) Lower Respiratory Tract and Thoracic Contents; (VI) and (VII) Digestive System; (VIII) Urogenital System and Adrenal; (IX) Female Generative System; (X) Intracranial Structures and Eye; and Section (XI) of Synonyms and Index. An additional section containing a fascicle on "Transmissible and Transplantable Animal Tumors" is to be inserted. The internal arrangement of all the individual fascicles consists of the same orderly design: name of tumor (with synonyms), definition, natural history, morphology, differential criteria, and pertinent bibliography; but each author is urged to "state what he believes to be the truth" as text to elucidate his own selected illustrations of the tumors of those organs about which his knowledge is preeminent.

Tumors of the Peripheral Nervous System. Section II. Fascicle 6. By ARTHUR PURDY STOUT, Columbia. 56 ills. Pp. 50. 60c.

The versatility of the cells as compared to the relatively small number of these tumors, mentioned by the author in his "Introduction," is effectively depicted.

Teratomas. Section III. Fascicle 9. By RUPERT A. WILLIS, Leeds, England. 45 ills., 1 color plate. Pp. 58. 50c.

An excellently compact and relevant account of a group of lesions that has always been a source of confusion.

Tumors of the Parathyroid Glands. Section IV. Fascicle 15. By BENJAMIN CASTLEMAN, Harvard. 53 ills., 3 color plates. Pp. 74. 65c.

Another exemplification of the clarity of the facets that compose the Atlas's compound view of the special pathology of neoplasia.

Tumors of the Cartoid Body and Related Structures (Chemoreceptor System). Section IV. Fascicle 16. By PHILIP M. LECOMPTE, Harvard. 23 ills., 1 color plate. Pp. 40. 45c.

This should be as valuable to surgeons as to pathologists, since the author stresses the application of his knowledge in this field to the clinical problems presented by the tumors of the carotid body.

Tumors of the Lower Respiratory Tract. Section V. Fascicle 17. By AVERILL A. LIEBOW, Yale. 249 ills., 3 color plates. Pp. 189. $1.25.

Justifiably the longest fascicle, it therefore naturally contains more occasions for dispute, such as the retention of the term "adenoma of the bronchus" for tumors that metastasize.

Tumors of the Mediastinum. Section V. Fascicle 18. By HANS GEORGE SCHLUMBERGER, Ohio State. 75 ills., 1 color plate. Pp. 88. 75c.

The illustrations are well balanced between x-ray and photomicrographs and pictures of gross specimens. The text is an equally well balanced survey of these heterogeneous lesions.

Tumors of the Adrenal. Section VIII. Fascicle 29. By HOWARD T. KARSNER, U.S. Navy, Bethesda. 47 ills., 5 color plates. Pp. 60. $1.00.

Though brevity is seldom to be deplored, more upon this subject is to be desired. Perhaps advantage may be taken of the loose-leaf format to publish addenda by the same author.

Tumors of the Breast. Section IX. Fascicle 34. By FRED W. STEWART, Cornell. 68 ills., 2 color plates. Pp. 114. $1.10.

The text is readable and often pertinently witty: "The most frequent precancerous lesion of the breast is a cancer of the opposite breast." The illustrations are particularly good.

Tumors of the Central Nervous System. Section X. Fascicles 35 and 37. By JAMES W. KERNOHAN and GEORGE P. SAYRE, Mayo Clinic. 126 ills. Pp. 129. 90c.

An impressive piece of work (tumors of the pineal gland are included) that is a major contribution to our knowledge.

Tumors of the Male Sex Organs. Section VIII. Fascicles 31b and 32. By FRANK J. DIXON and ROBERT A. MOORE, University of Pittsburgh and Washington University. 135 ills. Pp. 179. $1.50.

Admirably thorough in text and illustrations, this fascicle maintains the high standard of the others.

Tumors of the Thyroid Gland. Section IV. Fascicle 14. By SHIELDS WARREN and WILLIAM A. MEISSNER, Harvard. 82 ills., 1 color plate. Pp. 96. $1.75.

This is an excellent presentation of the neoplastic conditions of the thyroid by one who is deservedly judged to be an authority on the subject. It is clearly

written and holds the interest of the reader. The illustrations are exceptionally good.

A critical review of the amount of work represented by the above list of titles is impossible in the space feasible. The reviewer's purpose is frankly to acquaint readers with the existence and prospective growth of a publication that already reveals itself as specialized academic pathology at its best. Opportunities for dissent are of course given, for the vigor of the individual writers' opinions has wisely been encouraged. Undoubtedly, however, the *Atlas* is accomplishing its intent to standardize the nomenclature of neoplasms and to organize, both pictorially and verbally, what is essential to the "definition and diagnosis" of neoplasms. The practitioner, the surgeon and the pathologist, and all students of cancer, will be grateful for it and, incidentally, for the benevolent subsidizing that permits its low price. The investigator, too, will find it a valuable source of reference.

DALE REX COMAN

Department of Pathology
University of Pennsylvania

Heredity in Uterine Cancer. By D. P. MURPHY. Cambridge: Harvard University Press, 1952. Pp. 128. $2.50.

This small volume of the Commonwealth Fund series contains the results of a study on cancer in relatives of 201 women with cancer of the uterus, as compared to 215 control probands. The author concludes that hereditary factors affect the frequency of cancer of the uterus and obtains some indication that hereditary factors influence the occurrence of cancer at other sites, although no conclusion as to whether all these factors are specific for sites other than uterus was possible. The bibliography is exceptionally abbreviated, contains few recent references, and no consideration of the results of animal experimentation is included. It is also unfortunate that the sources of material did not allow distinction between carcinoma of cervix and corpus uteri and between different histological types of the malignancies studied. However it is apparent that a careful study has been made, and limitations are largely those of utilizing human material. One is impressed with how well the data are organized, and the generous allocation of space to explanation of methods used is commendable.

WALTER J. BURDETTE

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Das Phasenkontrastverfahren in der Medizin. By H. G. HANSEN, A. ROMINGER, and K. MICHEL. In German. Göttingen: Vandenhoeck & Ruprecht Verlag. Pp. 92.

The first part of this small manual on the physical aspects of phase-contrast microscopy introduces the reader to the theoretical side of this important tool for studying intracellular structures. This part is very clearly written. The practical side of this technic is dealt with in the second part. Here we find detailed instructions as to how to employ phase contrast microscopy in different fields.

Apart from a general introduction with respect to the preparation of objects to be studied, this part covers the use of phase contrast microscopy for the following fields, in particular: hematology, biopsy, normal and pathological histology, tumor cells in tissues and body fluids, uni- and multicellular organisms, normal and pathologically altered body fluids, and excreta.

This monograph contains numerous excellent photographs and drawings and can be highly recommended to those who wish to familiarize themselves with this research tool.

HANNS SCHMITZ

McArdle Memorial Laboratory
University of Wisconsin

Tendances Actuelles des Recherches de Chimiothérapie des États Cancéreux. By R. TRUHAUT. Chapter in "Actualités Pharmacologiques" 5 éme série. In French. Paris: Masson et Cie, 1952. Pp. 58.

This review of experimental cancer chemotherapy written in French appeared last year. As it was published in a volume that may not be generally available to investigators in this field, it seems desirable to call attention to the review.

The compounds ordinarily considered in such reviews have been discussed competently under two categories: (1) those agents which modify the cellular environment and (2) those acting directly upon the cell—e.g., mitotic poisons, antimetabolites, and miscellaneous substances. The material covered is now at least a year out of date, but that is the fate of any review in a still rapidly growing field. Despite this unavoidable defect the review is a useful presentation of the perspective of a European investigator and also has an excellent bibliography, which contains 319 specific references listed alphabetically plus a list of 50 general references and reviews. Some of the references are ones not observed in other reviews on this subject either because of their temporal or spatial unavailability.

Evidence for care in using some of the material in the review may be found in errors that happened to be noted—e.g., the formula of picropodophyllin, page 229, is incorrect even for what was considered to be the formula at the time the review was prepared; on page 231 melamine has been misspelled; on page 247 reference is made to Sarcoma 180 in the rat rather than the mouse; and in the bibliography Hoster's name has been misspelled. In spite of these minor errors the review should be quite useful to those interested in experimental cancer chemotherapy.

C. CHESTER STOCK

Division of Experimental Chemotherapy
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Voprosi Onkologii (*Problems of Oncology*). N. N. PETROV, A. I. RAKOV, A. I. SEREBROV, S. A. KHOLDIN, and L. M. SHABAD (eds.). In Russian. Moscow: Acad. Meditsinskikh Nauk SSSR, 1950. Pp. 536. Price: 37 roubles, 80 kopeks.

This is a publication of the proceedings of an All-Soviet Union Conference on Oncology, held in Lenin-

grad in January, 1947. It was attended by 1,220 individuals, of whom 915 were delegates.

The conference was divided into two major sections, on Theoretical Oncology, or research, and on Clinical Oncology. The latter had three panels, on cancer of the esophagus and stomach, on cancer of the uterine cervix, and on other malignant tumors, including cancer of the rectum, thyroid, and lip. There were delivered 44 papers.

In general, the proceedings reflected much the same thinking and directions as would have been recorded in a similar conference in the United States. There was an absence of chauvinism, Western workers were well recognized and referred to, and no political influences were obvious in the discussions.

No particularly new departures in cancer research were presented. Somatic mutation, viruses, changes in protein synthesis, and the attempt to unite carcinogenic fractions isolated from livers of cancer patients with the milk factor in mice into a theory of endogenous production of carcinogens were discussed. There were no papers on genetics of cancer, either in man or in animals. Only two unusual papers appeared, reflecting investigations that seem to be limited to the Soviet Union. One concerned the continued interest in the role of the reticuloendothelial system in cancer, by R. E. Kavstsky *et al.* (pp. 85–91) of Kiev and the other by B. S. Pesochensky (pp. 501–506) on mitogenetic radiation, a phenomenon not generally accepted as existent.

The reviewer was particularly interested in the following papers: L. M. Shabad and Z. V. Golbert (pp. 130–139) reported on the multicentric origin of cancer of the mammary gland in women. Multiple primary foci of cancer were found in 32 of 440 cases; in 23 additional cases precancerous lesions were found. S. S. Weil (pp. 140–155), in a paper on chronic nonspecific pneumonia and bronchogenic cancer, indicated that the continual rise in bronchogenic cancer was also seen in Russian statistics, at least in Moscow and in Leningrad; in 1944 and 1945 it accounted for 12.4 and 10.9 per cent of all cancer deaths, being the second most frequent cause of cancer mortality.

Several extensive surgical series were reported. A. G. Savinik (pp. 229–242) summarized data on 525 patients with cancer of the gastric cardia seen at the Tomsk Medical Institute between 1929 and 1946. Resection of the cardia, or total gastrectomy, was performed in 154 cases, or 29.3 per cent; since 1931 the approach has been through a sagittal resection of the diaphragm. Patients who had symptoms of dysphagia for less than 5 months were usually resectable. Unfortunately, follow-up was available in only 37 resected patients, of whom 22 were well, seventeen for 3 years or longer and fourteen for 5–9 years. In the discussion, V. V. Tatarsky (pp. 289–291) stated that of 2,542 patients with gastric cancer seen at the Leningrad Oncologic Institute between 1926 and 1945, 888, or 34.9 per cent, were resectable. B. V. Petrovsky (pp. 272–279) of Moscow presented a paper on the transpleural resection of the esophagus and cardia, including 22 personal cases with four postoperative deaths.

V. P. Michailov and A. A. Terechova (pp. 337–343) of Moscow presented data on the early and late results of 800 Wertheim operations for cancer of the uterine cervix. This included 113 cases in Stage I, 252 cases of Stage II, 385 cases of Stage III, and 150 cases of Stage IV. Primary operative mortality was 15.6 per cent. In 443 operations from 1924 to 1942, 71 died postoperatively, and 183 were lost to follow-up; 122 died of cancer and 67 were known to be alive and well 5–9 years postoperatively. M. P. Domshlak (pp. 323–331) of Moscow wrote on the radiation therapy of cancer of the uterine cervix. He quoted that Soviet literature between 1931 and 1940 indicated the following 5-year results: Stage I, 56.1 per cent; Stage II, 31.5 per cent; Stage III, 13.7 per cent; Stage IV, 0.5 per cent. This was compared to the League of Nations' report of 1938, showing 57.2, 37.6, 22.0, and 5.5 per cent, respectively. The results on 396 cases seen at the Central Oncologic Institute, 1934–1938, with the cases being distributed by Stages from I to IV at 46, 164, 159, and 27 in number were: 76 living and well at 5–10 years; 129 living and well at 1–4 years; and 191 dead or lost to follow-up.

S. A. Kholdin (pp. 423–439) of Leningrad discussed the operative treatment of cancer of the rectum; 925 cases were seen at the Oncology Institute between 1927 and 1941. The percentage of radically resectable cases had risen from 17.2 in 1927–31 to 27.7 in 1937–41. Postoperative deaths, on 353 cases from two institutions, were encountered in 18.1 per cent, with a drop from 23.9 per cent before 1935 to 13.6 per cent during more recent years. The late results of the procedures were not presented.

It is the impression of the reviewer that the surgical papers displayed evidence of considerable technical ability, as well as originality in extending operations for cancer of the esophagus, stomach, cervix, and rectum. Operative mortality was higher than reported from many clinical centers of the United States, but it was also apparent that the criteria for operability were less rigid. In contrast to the surgical papers, reports dealing with radiation treatment were mediocre, and there was little evidence of new technics or modern considerations of dosimetry; none of the presentations mentioned high voltage radiation. All clinical papers suffered from a high percentage of cases lost to follow-up, such loss being 40–60 per cent in most series; in part this loss was undoubtedly accountable to the dislocations occasioned by the war.

The conference had no specific papers on the organization of the cancer research or the clinical programs in the Soviet Union and concluded with a very general statement and a complete list of the delegates. The remarks suggested that oncology is considerably more accepted as a specialty in the Soviet Union than it is in the United States, and that a real attempt was being made to correlate more closely the clinical and the laboratory endeavors in the field. In all, the volume is a contribution to oncologic literature and contains a number of papers that would be of interest to specialists in specific fields.

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Announcements

Corrigendum

The following corrections should be made in the abstract by R. Schoental ("Primary Liver Tumors in Rats as a Result of Feeding of Senecio Alkaloids") in the *Proc. Am. Assoc. Cancer Research*, 1:47, 1953.

This work came from the Cancer Research Dept., Royal Beatson Memorial Hospital, Glasgow, Scotland. Histological evaluation of the liver changes were by Drs. P. R. Peacock and M. A. Head. The South African Senecio alkaloids which produced liver tumors were a gift from Prof. F. L. Warren, University of Natal, Pietermaritzburg.